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No. 1. SIMON FLEXNER, J. W. JOBLING, and MAUD L. MENTEN. Experimental studies on tumor. Plates 1–28. (Issued June 30, 1910.) Price, $1.00.

HUMAN GROWTH CURVE.

By CHARLES B. DAVENPORT.

(From the Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor.)

(Accepted for publication, July 26, 1926.)

1. Statement of Problem.

The fascinating changes in the velocity of development, as well as in proportion of parts, that the growing child shows have long been watched with interest by parents but have only lately been made the subject of scientific analysis. Quetelet (1870) was perhaps the first to measure children at each year of age; but his subjects were few in number (ten to each year), strictly, but not always wisely, selected. Then came the measurements of great numbers of school children by Bowditch (1875) in Boston. This work was followed by a host of similar investigations whose results are summarized by Baldwin (1921) and in my 1926 paper.

The first attempt to interpret the course of human development on a chemical basis was made in the same year by W. Ostwald and by T. Brailsford Robertson, the latter of whom has published a remarkable series of papers since 1908. Robertson early concluded that there are three maxima in the curve of growth of man; one intrauterine, a second that reaches its greatest velocity at about the 6th year, and a third which, in the male, occurs at about the 16th year. This view of a triple set of growth cycles is still adhered to by Robertson who discusses them fully in his book Chemical basis of growth and senescence (1923) and later papers. Robertson's conclusions have been largely based on the data published by Quetelet, and this selection has not been altogether fortunate. Brody has extended Robertson's methods of analyzing the growth curve; but recently (1926) he has found the human growth curve to be of a different order from the growth curve of other mammals and he has been led to abandon, for the present, attempts at its interpretation. In view of the un-

satisfactory condition of the analysis of the human growth curve it has seemed desirable to reattempt it, using the best available data. This is the excuse for the present paper.

2. Methods and Material.

The curve of development of weight from conception to maturity (Fig. 1) is based on data drawn from various sources. For the antenatal portion the data of Streeter (1920) have been utilized. For postnatal weights, up to 6 years, the data of Woodbury (1921) have

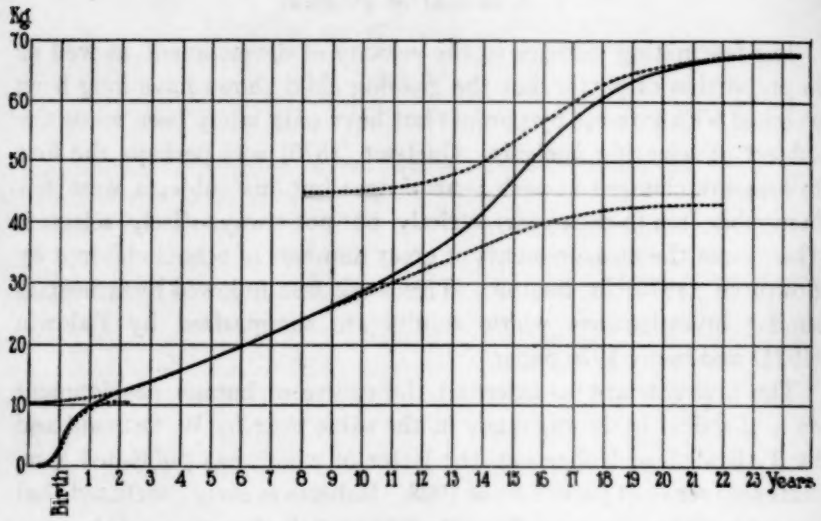

FIG. 1. Analysis of the developmental curve of body weight (full line) into two auto-catalytic curves (dotted line at top and bottom) and a residual curve (dotted line in the middle). Human Nordic stock, males. The autocatalytic and residual curves drawn in free-hand. Abscissæ, time in years; ordinates, body weight in kilos.

been used. For later years various sources, chiefly Nordic males as given in Table B of my Human metamorphosis (1926), were used.

For annual increments in weight (Fig. 2) the same sources have been used, together with my Table D (1926) for Nordic males.

The dotted curves of Fig. 1 were put in free-hand to indicate the location of possible autocatalytic curves. The dotted curves of Fig. 2

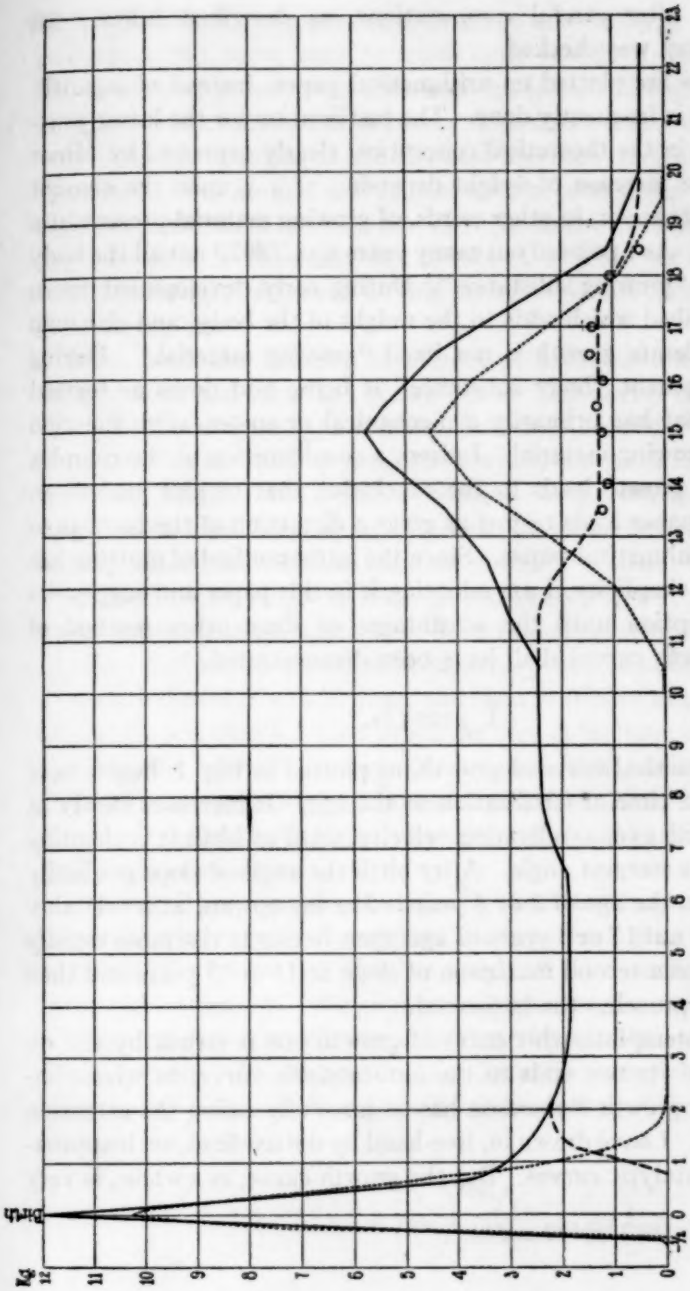

FIG. 2. Full line, the curve of annual increment rate of total body weight, male Nordic stock (see text). Dotted line to left, theoretical skew curve corresponding to increments of circumnatal growth cycle. Dotted line to right, theoretical "normal" curve of increments corresponding to adolescent growth cycle. Dash line, 0.75 to 2.25 years, full line 2.25 to 10.5 years, dash line 10.5 to 20 years, and full line beyond 20 years indicates the residual growth increments. Abscisse, time in years; ordinates, annual rate of increments in weight in kilos. Small circles between 13 and 19 years indicate the precise points upon which is based the position of the dash line, which is smoothed between these years.

were drawn after careful computations, as described below. All statistical work was checked.

The curves are plotted on arithmetical paper, instead of logarithmic paper as is frequently done. The justification for the latter practise is found in the theoretical conception, clearly expressed by Minot (1891): "The increase of weight depends . . . . upon the amount of body substance or, in other words, of growing material present at a given time." As I pointed out many years ago (1897)[1] not all the body substance is "growing substance." During early development much water is imbibed which adds to the weight of the body, and although it may accelerate growth is not itself "growing material." During later development "body substance" is being laid down as formed substance that has primarily a mechanical or sustentative function and is not growing material. Indeed, a consideration of the complex processes of growth leads to the conclusion that to plot growth on logarithmic paper leads to just as great a distortion of the facts as to plot it on arithmetical paper. Since the latter method of plotting has the merit of simplicity, I am adopting it in this paper and suggest its uniform adoption until the advantages of some other method of plotting growth curves shall have been demonstrated.

3. RESULTS.

The arithmetical curve of growth, as plotted in Fig. 1, begins near 0 kilos at the time of fertilization of the egg. It increases slowly at first, then with ever accelerating velocity, until at birth it is shooting upward at its steepest angle. After birth the angle of slope gradually diminishes to the age of 2 or 3 years. It runs upward at a tolerably uniform rate until 7 or 8 years of age, then begins to rise more rapidly again; reaches a second maximum of slope at 14 or 15 years and then gradually approaches the horizontal.

If one contemplates this curve of growth one is struck by the resemblance of its two ends to the autocatalytic curve, to whose importance for growth Robertson has so forcefully called the attention of biologists. I have drawn in, free-hand by dotted lines, such approximate autocatalytic curves. But the growth curve, as a whole, is very

[1] Davenport (1897), pp. 82 and 83.

far from being merely one, two, or three autocatalytic curves. The analysis of the curve may be made more readily if we transform it into a curve of growth velocities, and this is done in Fig. 2, which also is drawn on the arithmetical scale. The curve thus drawn is an instructive one. Starting at the zero base line the velocity of increments in weight rises, at first slowly, them more and more quickly, to a striking peak which corresponds with the moment of birth. At this time the child is increasing at the rate of 12 kilos per annum. The absolute velocity of growth at this period is greater than at any other time during life.

After birth the velocity of growth proceeds to diminish just as rapidly as it had increased before birth. This leaves out of account the cessation of growth that is well known to occur during the 2 or 3 days after birth, since to consider it would unnecessarily complicate the main result. The curve of velocity of growth in weight runs nearly on a level from $3\frac{1}{2}$ to $6\frac{1}{2}$ years. It then rises very gradually for a year or two, remains constant from $8\frac{1}{2}$ to about 10 years, and then proceeds to climb up to a second peak which it reaches at 15 years (in the male), at which time there is an annual growth increment of about 5.75 kilos per annum. After this peak is reached the velocity of growth diminishes to 20 years and then continues at an annual rate of slightly less than 0.5 kilos to middle life. The curve does not reach zero, on the average, because the population of adult males in the United States gains about 1 pound a year from 22 to 26 years and $\frac{1}{2}$ pound thereafter until about 45 years and about $\frac{1}{3}$ pound from then until 55 years of age.

Our velocity curve brings out clearly the fact that growth is not one autocatalytic process. It suggests the hypothesis that there are two autocatalytic cycles; one that we may call the circumnatal and the other the adolescent. The circumnatal cycle begins at fertilization of the egg; reaches a maximum at birth and probably ends at between 2 and 3 years. An attempt to fit a theoretic curve to this cycle has been made. The best fit is given by a skew curve of Pearson's (1895) Type I. Its formula is:

$$y = 854.9\left(1 + \frac{x}{5.221}\right)^{0.985}\left(1 - \frac{x}{38.876}\right)^{7.334}$$

This curve is plotted by a dotted line in Fig. 2. Its standard deviation is only 5.17 months. The y_0 value is 854.9 gm. per month or 10.259 kilos per year. This falls short of the empirical value of 12 kilos per annum partly because smoothed values were used in computing the theoretical curve. The modal velocity of 12 kilos at birth is based on Zangemeister's data (1911), which are at least conservative in respect to velocity of growth at birth.

The circumnatal curve of velocity is, as stated, a skew curve of Pearson's Type I. The index of skewness is 0.31. In the formula given above the denominator of the fraction in the second factor gives the range in months of that part of the curve that lies between birth and conception. It is 5.22 months. The denominator of the fraction in the third factor gives the range in months of that part of the curve that lies between birth and the end of the circumnatal growth spurt, amounting to 38.88 months. The theoretical range to the left of the mode is thus only 5.2 months, while, actually, development begins at 9 months before birth. However, at the end of the 6th month before birth increments in weight are only just becoming considerable (10 gm. monthly) so that the calculated curve agrees here fairly well with the observed curve. The other end of the curve is at about 39 months, at which age the observed curve of increments has nearly reached the bottom of its first peak. Thus the theoretical and observed curves are in close agreement.

The adolescent spurt is probably measured by a normal frequency polygon, whose mode at 15 years in the male corresponds with that of the total increment curve. It seems to start at about the 10th birthday and ceases at the 20th. The adolescent episode of growth thus extends over 10 years or from 10 to 13 per cent of the full span of life.

The theoretical normal curve that most clearly accords with the observed adolescent curve is shown at the right of Fig. 2 in the dotted line. There is assumed a substratum of generalized growth which, after 12 years of age, gradually declines from 1.9 kilos per annum to 0.5 kilos. The theoretical curve is drawn in accordance with the formula

$$y = \frac{n}{\sigma\sqrt{2\pi}} e^{-1/2\left(\frac{x}{\sigma}\right)^2}$$

where n = 19,000 kilo-years; σ = 1.70 years, and y_0 = 4.47 kilos.

After subtracting the two special curves from the general curve of increments there remains a residual curve. This begins at somewhere about 9 months after birth; rises to the level of the total curve, and coincides with it during the period from 3 to 10 years. That the residual curve does not start at conception would not justify the conclusion that there is no basal growth occurring, independent of the circumnatal spurt, but indicates only that the circumnatal spurt is of such high velocity and that of basal growth is of such low velocity that the latter is quite obscured by the former. From 3½ to 6 years growth is apparently entirely residual and it is very steady and constant, at about 1.85 kilos per annum. There is a slight rise during the 7th year of life to a new constant velocity of growth of 2.4 kilos per annum. This rate of growth continues until 11.5 years is reached at which time the adolescent spurt has already started. The basal increment now diminishes rapidly as the adolescent spurt speeds up. It remains at about the 1.225 kilo level of annual increment from 13.5 to 17.5 years of age and then falls away to the constant rate of 0.45 kilos per year which is reached at 19 years. Statistics gathered by insurance companies (Medical Actuarial Mortality Investigation (1912)) indicate that weight increases, in the male of average stature, about 1 pound a year from 20 to 26 years and then about ½ pound yearly to 45, as stated above.

4. DISCUSSION.

The early optimism as to the possibility of resolving the total growth curve of man into three "growth cycles" superimposed upon one another (Robertson (1923)[2]) has given way to the recognition of the great complexity of this growth curve. Thus Brody (1926)[3] states that "the smoothed time curve of growth in weight [presumably of mammals, in general] is sigmoid, but the point of inflection, or rather region of inflection, is not in its center but where slightly over one-third of the mature weight is reached. The growth curve of man is the only exception encountered." Now our data show two points of inflection that clearly approximate the logistic curve. One occurs at birth and one at 15 years, in the male. Our data do not reveal the S-shaped

[2] Robertson (1923), p. 446.
[3] Brody (1926), p. 235.

curve at "the third, fourth and fifth years" which Robertson finds (1923)[4], and we fail to find in Robertson's or Brody's papers any sufficient evidence of this third or "juvenile" cycle. (Compare my discussion of this matter (1926).[5])

What do the facts, as revealed in the curve of velocity (Fig. 2), show clearly? Besides the circumnatal and adolescent growth cycles there is a mass of growth of irregular velocity from 2 to 10½ years, in the male, which tails away toward 20 years but continues on to 50 years of age, or later. This residual curve does not fall into any autocatalytic cycle. The existence of growth outside of "the three growth cycles" has been recently recognized by Robertson (1926)[6] who introduces the idea and the term of "linear increment." In the mouse he conceives this to begin at about 10 weeks after birth and to increase in arithmetical fashion to 140 weeks, or later. This "linear increment" conception was forced from the fact that growth of mice "continues very slowly for long after the attainment of sexual maturity and dimensions which might readily be supposed to be 'adult' and, therefore, maximal. It is possible that in other animals also a similar linear accretion is occurring, and has escaped attention for lack of data concerning the late growth of the animals."

Now I suspect that the residual curve indicated by the dash line and, in part, by a full line in Fig. 2, corresponds to Robertson's "linear accretion," inasmuch as it continues past maturity; but in detail it is entirely different from Robertson's "linear accretion" since it is not a straight line at all.

One may propose a hypothesis as to the meaning of this residual curve. One may base it on the probability that besides the natal and adolescent growth accelerators there are other growth processes of particular organs or of the body as a whole. These constitute the substratum of growth of which the natal and the adolescent cycles are especially activated or accelerated episodes. Indeed, it is plain from such studies as Riddle (1925) has made on the growth of organs in the pigeons and which Scammon (1925, 1926, *a*, *b*) is making on the growth of organs in man, that the total growth is, as it were,

[4] Robertson (1923), pp. 445 and 446.
[5] Davenport (1926), pp. 210–212.
[6] Robertson (1926), pp. 469–473.

the summation of growing parts or organs, each following a more or less independent law. Not until we understand the changes in weight of the different parts of the body from the beginning of development to maturity shall we be enabled to give an adequate interpretation of the growth curve. When that happens we shall first be in a position to direct and modify the form of the developmental curve.

To illustrate, merely, the view of an important substratum of growth apart from the cycles I may refer to the findings in respect to the rate of development of three or four human organs in comparison with the growth of the body as a whole.

Thus Starkel and Wegrzynowski (1910) and E. Thomas (1911) find that the suprarenals grow rapidly in the fetus, attaining, at or about birth, a weight of 3 gm. After birth the weight falls, absolutely, to about 1.5 gm. at about 12 months of postnatal life. It then increases very slowly to about 3 gm. at about the end of 5 years. Thomas shows that the degeneration after birth affects, especially, the deeper layers of the cortex. Scammon (1926, *b*)[7] shows, in addition, that in the suprarenals there is no extraordinary prenatal acceleration of growth but only a postnatal involution. A similar postnatal retardation of growth-velocity occurs in the cerebellum (Scammon and Dunn (1924)).

The length of the uterus in the fetus undergoes extraordinary changes that have been worked out by Scammon (1926, *a*).[8] Thus in the 7th fetal (lunar) month the uterus begins to show an extraordinary spurt in growth, as compared with the body as a whole. At birth the length of the uterus is 35 mm. while, had the spurt not occurred, it would have been only about 23 mm. Within 3 weeks after birth the length of the uterus has fallen to 24 mm; and then increases slightly during the following 5 months. "This suggests," says Scammon, "that the growth of the uterus in the latter fetal months consists of a substrate of typical fetal growth plus a secondary growth increment, which, presumably, is due to an extra stimulus furnished by a hormone of placental or possibly ovarian origin. After birth the organ loses this secondary increment but retains that result-

[7] Scammon (1926, *b*), p. 809.
[8] Scammon (1926, *a*), p. 690.

ing from the early fetal growth rate." Again, reference may be made to the well known case of the thymus, which, according to Hammar (1921),[9] undergoes a rapid reduction of size and function as adolescence sets in at 11 to 15 years. This involution seems to be determined and controlled by the development of the gonads.

The foregoing interesting studies on variations in the velocity of growth of human organs justify the conclusion that the development of weight in man is the resultant of many, more or less elementary, growth processes. When some special activator of development causes one or more organs simultaneously to increase in velocity of growth to a high degree then a marked maximum occurs in the human growth curve, and this may assume the form of the logistic curve of growth. Two of these episodes are of overwhelming importance. The great number of smaller growth operations are less outstanding, and overlap in time to such a degree as to become submerged in a nearly uniform, high and prolonged wave of growth. It is probable that some of these growth impulses affect not merely one or two organs of the body but are diffused more or less uniformly throughout the entire body. It is this substratum of the growth process which deserves special study and analysis in the future.

Finally, one is led to speculate on the nature of the activators of the two principal special growth accelerations—the circumnatal and the adolescent. There is some ground for entertaining the hypothesis that the adolescent spurt is especially activated by the secretions of the pituitary gland, or anterior lobe of the hypophysis; since preadolescent hypophyseal underactivity results in reduced growth and preadolescent hyperactivity in giant growth.

The tremendous velocity of growth in the circumnatal cycle seems to be activated by something coming into the fetus from the mother through the placenta. Hardly otherwise can we account for the fact that the growth process ceases its acceleration at just the time when the placental connection is broken.

Experiments should throw light on the nature of the special, as well as the general, growth activators at different stages of development.

[9] Hammar (1921), p. 551.

5. SUMMARY OF CONCLUSIONS.

The human growth curve shows two (and only two) outstanding periods of accelerated growth—the circumnatal and the adolescent.

The circumnatal growth cycle attains great velocity, which reaches a maximum at the time of birth. The curve of this cycle is best fitted by a theoretical skew curve of Pearson's Type I. It has a theoretical range of 44 months and a standard deviation of 5.17 months. The modal velocity is 10.2 kilos per year.

The adolescent growth cycle has less maximum velocity and greater range in time than the circumnatal cycle. The best fitting theoretical curve is a normal frequency curve ranging over about 10 years with a standard deviation of about 21 months and a modal velocity of 4.5 kilos per year.

The two great growth accelerations are superimposed on a residual curve of growth which measures a substratum of growth out of which the accelerations arise. This probably extends from conception to 55 years, on the average. It is characterized by low velocity, averaging about 2 kilos per year from 2 to 12 years. It is interpreted as due to many growth operations coincident or closely blending in time.

Our curve shows no third marked period of acceleration at between the 3rd and 6th years.

The total growth in weight of the body is the sum of the weight of its constituent organs. In some cases these keep pace with the growth of the body as a whole; great accelerations of body growth are due to great accelerations in growth of the constituent organs. In other cases one of the organs of the body (like the thymus gland) may undergo a change in weight that is not in harmony with that of the body as a whole.

The development of the weight in man is the resultant of many more or less elementary growth processes. These result in two special episodes of growth and numerous smaller, blending, growth operations.

Hypotheses are suggested as to the basis of the special growth accelerations.

I take this occasion to acknowledge the valuable assistance of Miss Mary T. Scudder in the calculation of the two theoretical curves of Fig. 2.

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THE EFFECT OF ENZYME PURITY ON THE KINETICS OF TRYPTIC HYDROLYSIS.

By HENRY BALDWIN MERRILL.

(From the Laboratory of A. F. Gallun and Sons Company, Milwaukee.)

(Accepted for publication, August 28, 1926.)

INTRODUCTION.

In the course of an investigation of the function of pancreatic enzymes in the tannery process known as bating, the author has had occasion to study the rate of digestion of keratose by trypsin.[1] This study has resulted in the accumulation of a considerable mass of data bearing on the kinetics of the reaction under different experimental conditions. Examination of these data from the point of view of the law of mass action has brought out an interesting relation between the purity of the enzyme specimen employed and the apparent order of the reaction. It appears from this work that the less refined the enzyme, the more closely does the digestion follow the course of a monomolecular reaction. The evidence for this finding, and its bearing on the mechanism of the inactivation of trypsin, form the subject matter of this paper.

Keratose.—Since this material has not previously been studied as a substrate for enzyme action, a brief description is called for. Keratose (to apply a general term to what may or may not be a group of substances) is the first product of the alkaline hydrolysis of keratin. In its physical chemistry, keratose resembles casein, being soluble in dilute acid or alkali, but insoluble at its isoelectric point, which has been found to lie at pH = 4.1.[1] The method used in this laboratory for preparing keratose[2] consists of dissolving clean calf hair in dilute sodium hydroxide, neutralizing to pH = 8.0, filtering off any undecomposed hair, precipitating keratose in the filtrate at its isoelectric point, and washing repeatedly by decantation. The white, curdy

[1] Wilson, J. A., and Merrill, H. B., *Ind. and Eng. Chem.*, 1926, xviii, 185.

[2] Wilson, J. A., and Merrill, H. B., *J. Am. Leather Chem. Assn.*, 1926, xxi, 2, 50

precipitate so obtained is redissolved in dilute NaOH, and brought to pH = 8.0, which has been found to be the pH value at which tryptic hydrolysis of keratose is most rapid.[1]

Enzymes.—The enzymes used in this work were commercial samples submitted to this laboratory for test as possible bating materials. They were used without any purification. The samples varied in strength from a U. S. P. pancreatin, the activity of which, measured on casein, was 7 Fuld-Gross units, to a purified trypsin of 333 Fuld-Gross units. Out of all the samples examined, three, representing respectively a very weak, a moderately strong, and a very strong preparation, were employed for the work covered by this paper. The characteristics of these samples were as follows:

	Activity measured on	
Sample No.	Casein (Fuld-Gross units).	Keratose[2] (Wilson-Merrill method).
9	333	133
6	83	27.3
2	7	4.2

EXPERIMENTAL.

Method.

The experimental method employed in studying the rate of digestion of keratose by enzymes is very similar to that used by Northrop[3] in his work with casein. The method is based upon the fact that keratose, like casein, is insoluble at its isoelectric point, while its products of digestion are soluble. Starting with a known quantity of keratose, the fraction remaining undecomposed at the end of any given time may be determined gravimetrically, and the quantity of keratose digested determined by difference. This method has the great advantage, as pointed out by Northrop, that only the first step of the digestion is studied.

The stock solution of keratose is analyzed by precipitating a measured volume at pH = 4.1, filtering through tared filter paper, drying at 100°C., and weighing. From the analytical results, the volume of stock solution containing exactly 2.000 gm. keratose is calculated. This volume is placed in a liter flask with 100 cc. of the powerful citrate-phosphate-borate buffer solution (pH = 8.0) described by Northrop, and made up nearly to 1 liter. The flask is placed in the thermostat and allowed to come to the desired temperature; then the enzyme, dissolved in a little water, is added, the solution is made up to 1 liter, and well

[3] Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 264.

shaken. 100 cc. aliquots are removed immediately after adding the enzyme, and at suitable time intervals thereafter. The undigested keratose is precipitated at pH = 4.1 by running each aliquot into 50 cc. of a sodium acetate-acetic acid buffer, pH = 3.6, N/2 in acetate ion. The precipitate is allowed to settle, filtered through a tared paper, washed four times with very dilute HCl (pH = 4.1), dried, and weighed. The difference between the initial weight of keratose and that obtained after any given time interval gives the weight of keratose digested in the interval.

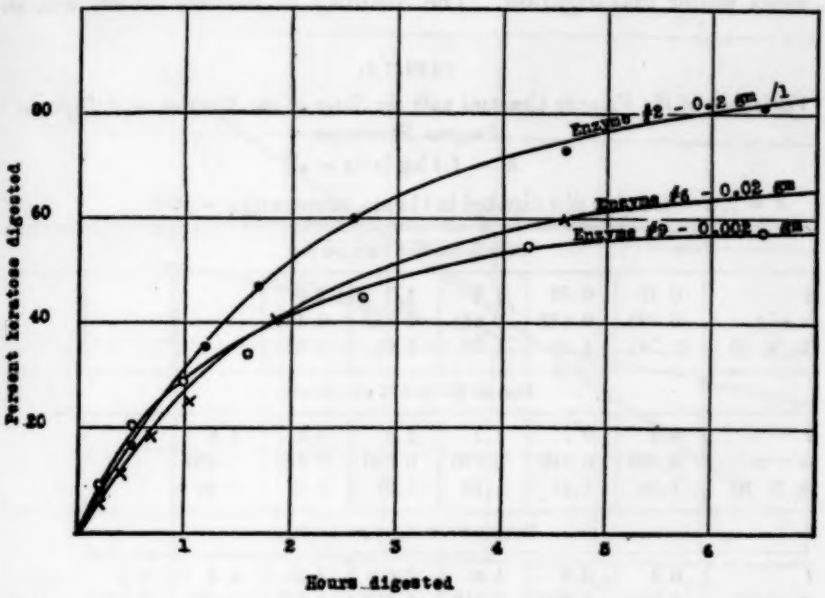

FIG. 1. Rate of digestion of keratose with different enzymes. 2.0 gm. keratose per liter, pH = 8.0, T = 35°C.

Suitable blanks were run, and corrections made for insoluble matter added with the enzyme, and for keratose digested in the absence of the enzyme. This last correction is negligible at the temperature employed for these experiments.

The temperature employed was 35°C., ± 0.05°.

Tests showed that the buffer employed kept the pH value constant to within 0.1 pH unit during the course of the digestion.

Bacterial action was inhibited by the use of thymol.

Calculations.—The per cent of the total keratose digested in different time periods was plotted against time for each series, and a smooth curve drawn through the points. Fig. 1 shows a set of

such curves, obtained with the three enzymes employed in this work. It is seen that most of the points lie on or close to the curves, indicating that the percentage error is small for work of this type.

The percentages digested at appropriate time periods were read off the smoothed-out curves, and employed in the calculations.

Order of Reaction.—By the method outlined above, the rate of digestion of keratose was determined, using the three enzyme specimens under investigation. The quantity of enzyme added was so

TABLE I.

Variations of the Velocity Constant with the Stage of the Reaction with Different Enzyme Specimens.

$k_1 = 1/t \log [a/(a - x)]$

$a = 1$, x = fraction of a digested in t hours, temperature = 35°C.

Enzyme No. 2—0.2 gm. per liter.								
t	0.27	0.50	1.2	1.7	2.6			
$a - x$	0.920	0.832	0.646	0.532	0.401			
$k_1 \times 10$	(1.34)	1.60	1.58	1.61	1.53			
Enzyme No. 6—0.02 gm. per liter.								
t	0.3	0.7	1.2	2.0	3.0	4.6		
$a - x$	0.910	0.810	0.720	0.610	0.510	0.400		
$k_1 \times 10$	1.36	1.31	1.19	1.07	0.97	0.86		
Enzyme No. 9—0.002 gm. per liter.								
t	0.2	0.5	1.0	2.0	3.0	4.0	5.0	6.0
$a - x$	0.900	0.800	0.710	0.610	0.535	0.485	0.450	0.430
$k_1 \times 10$	2.3	1.9	1.5	1.1	0.91	0.78	0.69	0.61

adjusted that digestion took place at approximately the same rate in all three cases. The experimental data was plotted (Fig. 1), and values for the per cent keratose digested were read off at appropriate time intervals. The velocity constant of the reaction was calculated, using the equation for a monomolecular reaction

$$k_1 = 1/t \log [a/(a - x)]$$

placing $a = 1$ and $x =$ the fraction of the total keratose digested in t hours. Briggsian logarithms were used.

The results of these calculations are presented in Table I, and are plotted in Fig. 2. It will be seen that for Enzyme No. 2, the weakest enzyme employed, the values obtained for k_1 are practically constant during the first 60 per cent of the reaction. In other words, the digestion does follow the course of a reaction of the first order. With No. 6, which is some 7 times as strong as No. 2, the values obtained for k_1 drop off rapidly, and with No. 9, a preparation having 30 times

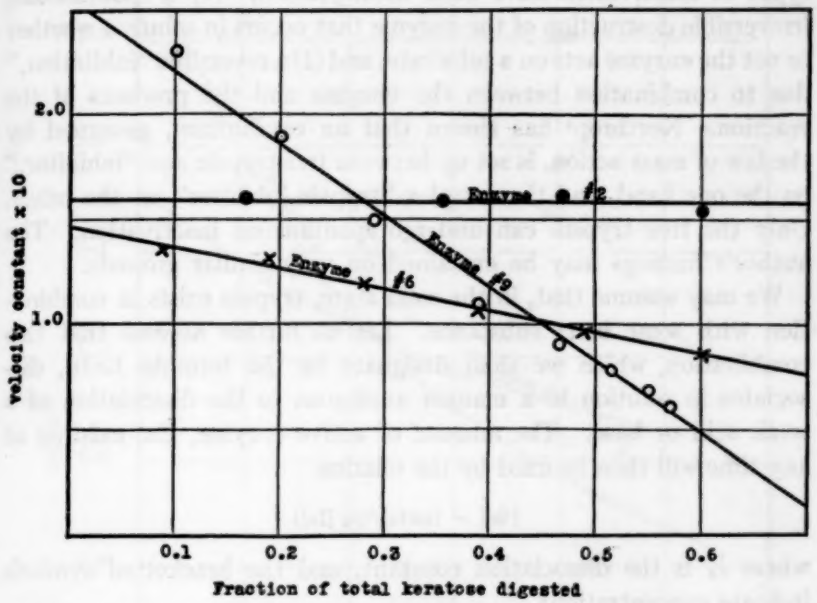

FIG. 2. Variation of velocity constants with stage of reaction for different enzymes.

the activity of No. 2, the rate of decrease of k_1 is very rapid,—the "constant" falling off to about one-fourth of its initial value when the reaction is little more than half completed.

In Fig. 2, k_1 is plotted as a function of the fraction of substrate digested. For the ideal reaction of the first order, the plot would be, obviously, a horizontal line. This condition is closely approached with Enzyme No. 2. With No. 6, and still more with No. 9, the departure of k_1 from constancy is sufficiently apparent.

It is significant that the plots of k_1 as a function of *fraction decomposed* are straight lines. This means that k_1 decreases in value in proportion to the amount of proteose hydrolyzed, and not in proportion to the length of time elapsed since the beginning of the reaction.

Inactivation of Trypsin.—The failure of an enzyme reaction to obey the law of mass action is commonly ascribed to progressive inactivation of the catalyst while the reaction is taking place. Two types of inactivation have been distinguished,—(1) a spontaneous, irreversible destruction of the enzyme that occurs in solution whether or not the enzyme acts on a substrate; and (2) a reversible "inhibition," due to combination between the enzyme and the products of the reaction. Northrop[4] has shown that an equilibrium, governed by the law of mass action, is set up between free trypsin and "inhibitor," on the one hand, and the complex "trypsin-inhibitor" on the other. Only the free trypsin can undergo spontaneous inactivation. The author's findings may be explained on very similar grounds.

We may assume that, in the solid state, trypsin exists in combination with some inert substance. Let us further assume that this combination, which we shall designate by the formula EnIn, dissociates in solution in a manner analogous to the dissociation of a weak acid or base. The amount of active enzyme, En, existing at any time will then be fixed by the relation

$$[\mathrm{En}] = [\mathrm{EnIn}]/(k_d\,[\mathrm{In}])$$

where k_d is the dissociation constant, and the bracketted symbols indicate concentrations.

Let us further assume that during purification of an enzyme, the concentration of active enzyme with respect to the inactive carrier is greatly increased. In a very impure preparation, [In] will be large, and [En] correspondingly small. The preparation will then be one of low activity. On the other hand, the undissociated complex, EnIn, will serve as a reservoir for En, more active enzyme being liberated as that present initially is used up by inactivation or combination with the products of the reaction. Thus [En] will remain practically constant during the course of the digestion. These are

[4] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 227, 245, 261.

the conditions which exist when a very weak enzyme, such as No. 2, is used for protein digestion, and, as we have seen, the constancy of enzyme concentration during the course of the reaction is reflected in the closeness with which the hydrolysis follows the course of a first order reaction.

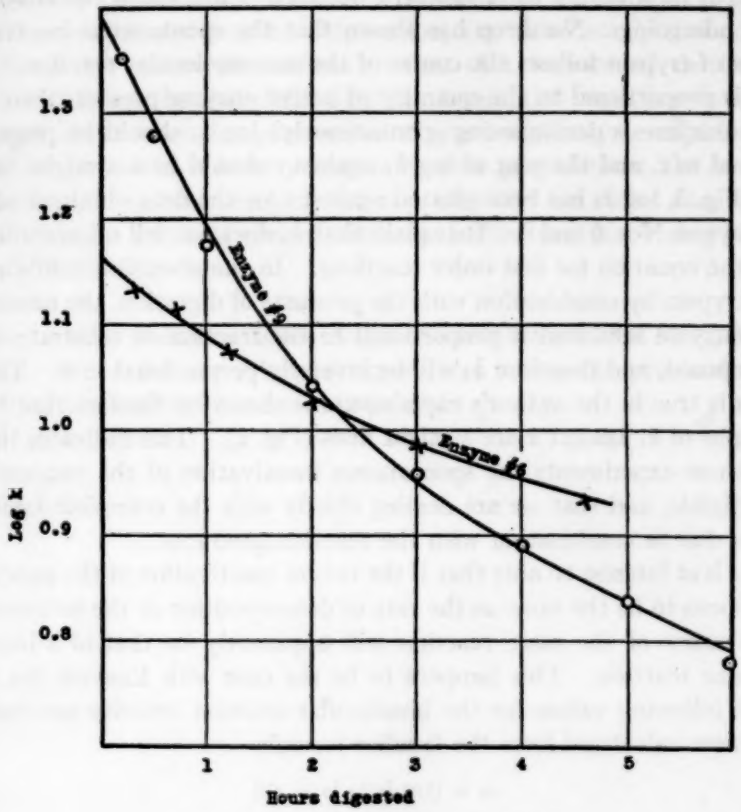

FIG. 3. Log k as a function of time.

In a highly purified enzyme, where the concentration of En is high with reference to the inert material, the complex EnIn will be largely dissociated at the start, and the preparation will manifest high activity. The free enzyme will, however, be inactivated or inhibited rapidly, and since no sufficient reservoir of combined enzyme

exists, the concentration of active enzyme will diminish. Under such conditions, the rate of hydrolysis of the substrate must fall off more rapidly than would be predicted from the equation for a first order reaction. Such is the case with Enzyme No. 9.

The manner in which the velocity constant of the reaction falls off affords information as to the type of inactivation which the enzyme is undergoing. Northrop has shown that the spontaneous inactivation of trypsin follows the course of the monomolecular reaction. If k_1 is proportional to the quantity of active enzyme present, then (if the enzyme is decomposing spontaneously) log k_1 should be proportional to t, and the plot of log k_1 against t should be a straight line. In Fig. 3, log k_1 has been plotted against t for the data obtained with Enzymes Nos. 6 and 9. It is plain that k_1 does not fall off according to the equation for first order reactions. In the reversible inhibition of trypsin by combination with the products of digestion, the amount of enzyme inhibited is proportional to the fraction of substrate decomposed, and therefore k_1 will be inversely proportional to x. That this is true in the author's experiments is shown by the fact that the graphs of k_1 against x are straight lines (Fig. 2). This indicates that in these experiments the spontaneous inactivation of the enzyme is negligible, and that we are dealing chiefly with the reversible inhibition due to combination with the reaction products.

It is of interest to note that if the rate of inactivation of the enzyme happens to be the same as the rate of decomposition of the substrate, the course of the main reaction will apparently be that of a bimolecular reaction. This happens to be the case with Enzyme No. 6. The following values for the bimolecular reaction velocity constant, k_2, were calculated from the familiar formula

$$k_2 = [1/t]\,[x/(a\,[a - x])]$$

from the data given in Fig. 1.

t	0.3	0.7	1.2	2.0	3.0	4.6
$a - x$	0.91	0.81	0.72	0.61	0.51	0.40
$k_2 \times 10$	3.3	3.3	3.2	3.2	3.2	3.3

With Enzyme No. 2, the corresponding values for k_2 increase as the hydrolysis proceeds, while with No. 9 they fall off, indicating in the

latter case that the enzyme is being used up at a relatively greater rate than the substrate.

SUMMARY.

The rates of digestion of keratose have been determined with three commercial enzymes, ranging widely in strength. It has been found that the weaker the enzyme preparation, the more nearly does the course of the hydrolysis conform to that of a reaction of the first order. This has been explained on the assumption that in solution an equilibrium exists between active enzyme, and enzyme combined with inert material. In very impure enzyme preparations, the large quantities of combined enzyme act as a reservoir for active enzyme, maintaining a constant concentration of active enzyme during the course of the digestion.

The author wishes to acknowledge his indebtedness to Mr. John Arthur Wilson for many helpful suggestions during the preparation of this paper, and for permission to publish.

TEMPERATURE AND FREQUENCY OF HEART BEAT IN THE COCKROACH.

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I.

It has been found that the relation existing between temperature and the frequency of rhythmic neuromuscular activity, especially when derived from many careful measurements made upon single animals at short intervals over a fairly wide range of temperature (Crozier and Federighi, 1924–25), may throw light on the question of the identity of the chemical reactions supposed to control homologous biological processes (Crozier, 1925–26, *b*). The present paper reports experiments performed further to test this hypothesis by determining the temperature characteristic for the frequency of the heart beat in *Blatta orientalis* L. Observations were made from large nymphs[1] of this species. All were secured in one lot from Birmingham, Alabama, early in 1926, and were kept at room temperature, with sufficient air, in a wire cage, where food (such as raw potato, butter, breadstuffs, and dead cockroaches) was available.

The data obtained possess added value in that the animals providing them remained whole and uninjured during the experiment. A 1000 watt lamp in a stereopticon lantern proved adequate to reveal clearly the pulsations of the dorsal blood vessel even in very darkly pigmented specimens, as viewed by the transmitted light with a low-power binocular microscope. (The absence of wings in the nymphs was favorable to this procedure.) The animal under observation, 22–26 mm. in length, was held in a glass tube, 30 cm. long and of such diameter as to press slightly upon the lateral edges of the terga. Wire gauze plugs, surfaced with cotton and set in place by wires

[1] Since one individual of the size used assumed wings at the next moult, these were presumably in the last nymphal instar.

attached to them, served to prevent movement forward or backward in the tube. The latter was connected at one end by way of rubber tubing to a suction pump, and at the other opened indirectly to the air of the laboratory; with the enclosed cockroach it was held immersed in the water of a rectangular thermostat. The bulb of a sensitive thermometer (graduated to 0.1°C.) was enclosed in air within a length of glass tubing of the same cross-section as that of the tube holding the animal, and was held close to the latter in order to approximate the same conditions. The light from the lantern, after passing through infra-red filters, was admitted to the thermostat only through an opening in a shield covering the exposed side. Here a lens concentrated the rays upon the ventral surface of the animal. A second shield of metal, fitted to the tube and with a small opening admitting illumination only to the thoracic sterna, protected the eyes of the observer from the glare and reduced photic excitation of the animal. A larger opening in the shield on the opposite side of the tube exposed to view most of the dorsum of the cockroach. Besides thus partly shielding the animal, further unnecessary photic stimulation was avoided by switching off the lamp between sets of readings.

The time for nine complete heart beats was determined with a stop-watch. As the several chambers pulsate almost synchronously in the normal cockroach, and at least at the same rate, that thoracic or (first, second, or third) abdominal segment was observed which during a given reading seemed most favorably situated. For every temperature step in a series, at least three, generally five, and occasionally up to eleven separate counts were made with a view to compensating the variations. Thus the present material represents somewhat more than 1500 stop-watch readings. In general these refer to the animal in a quiescent state; but for some sets it was virtually impossible to obtain (as was regularly attempted) complete counts without some coincident movements of body or legs. This probably did not greatly alter the average times recorded, or more in one sense than the reverse, for the consequent increase or decrease of heart rate (generally the former, but often apparently first one and then the other) seemed to be compensated in the course of two or three counts, possibly because of a fairly rapid rhythm of fluctuation (*cf.* Crozier and Stier, 1924–25). Although movements of the alimentary tract induced no

obvious change in the cardiac rhythm, I largely avoided making counts while such movements were more agitated.

The temperature was changed in steps of about 1°, rarely as great as 2°. After each such change a minimum of 10 minutes was allowed for adjustment before the next set of readings was taken. A portion of this interval passed while the air within the tube acquired the temperature of the thermostat, but during the last 5 minutes or more the mercury remained practically constant at the new level. The temperatures were read to 0.01°; in but a few exceptional cases did they vary as much as 0.2° from the first to the last of any single set of readings, and they are easily correct to ± 0.1°.

At each change of temperature (10 to 25 minutes before each set of readings), fresh air, passing first through nearly 1 m. of thin glass tubing in the thermostat, was drawn through the tube holding the cockroach. This procedure was a more than ample check upon disturbances which a change of oxygen or carbon dioxide tension might cause. In this connection I cite the remarkable case of Cockroach 10 which remained alive 29 days continuously in the tube. Its heart rate was approximately unchanged during more than 10 days, even with experimental exposure to 38.4° (once) and to 5° (twice); after each experimental series, air was drawn through the tube, but not oftener than every 8 to 48 hours.

II.

Satisfactory series of observations were completed with six individuals.[2] The data are transcribed in Figs. 1, 2, 3, and 4, where the logarithm of the frequency of the heart beat has been plotted against the reciprocal of the absolute temperature. The points represent the averages of the several readings made at each temperature step in a "run". It is apparent that within narrow limits of variation all the series describe straight lines. The relation of frequency to temperature therefore fits the Arrhenius equation $K_2/K_1 = e^{(\mu/R)\,(1/T_1 - 1/T_2)}$, in which K_1 and K_2 are proportional to velocity constants at the respective absolute temperatures T_1 and T_2, R the gas constant,

[2] Three other individuals were previously observed, in the course of developing and testing the experimental method. They yielded data not inconsistent with those about to be discussed.

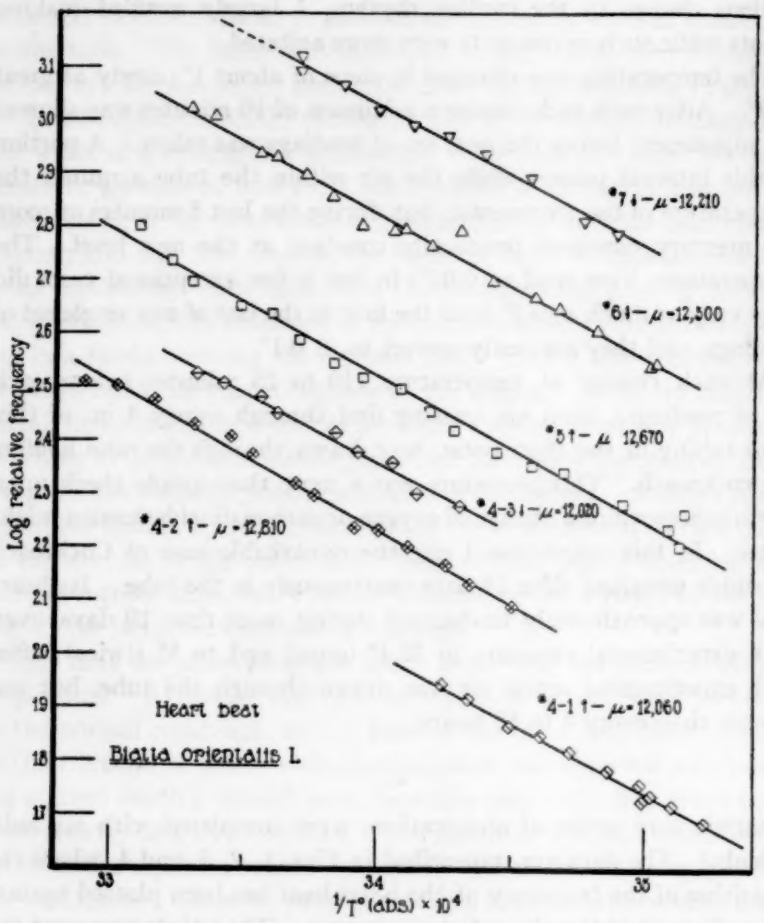

FIG. 1. Series of observations with Animals 4, 5, 6, and 7. Arrows indicate whether the temperature was raised or lowered. The frequencies are factored to raise their logarithms in each of the upper five series respectively by 1.0, 0.8, 0.6, 0.4, and 0.2 above the original values. The latter do not differ between series more than about 0.1 unit at any one temperature. The size of the symbols is such as to correspond to a probable error of ± 2 per cent of each average frequency, which is greater than the probable error found by calculation. The points marked by broken line symbols in the uppermost series were obtained before the animals had become adequately accustomed to the high temperature; hence the line was fitted to the rest of this series without considering them. Such deviations illustrate the necessity for careful thermal adaptation.

and μ the critical thermal increment or temperature characteristic (Crozier, 1924–25). The value of μ is fixed by the slope of the line best fitted to the points plotted; it may be computed from the graph, the frequencies being measures of the velocity constants.

Separate and repeated fittings to the series obtained from five individuals, shown in Figs. 1 and 2, lead to values of μ ranging from 12,020 to 12,810 calories for temperatures between 10° and 38°.[3] The weighted average μ = 12,500. Of the five animals one was cooled below 10°; two series from 19° to 5° show a "break" or marked change of temperature characteristic occurring at about 10°, such that for lower temperatures μ is of the order of 18,100 (Fig. 2).

Fig. 3 is a mass plot of the series (above 10°) which are shown in Figs. 1 and 2, but adjusted by suitable factors to coincide at $1/T$ = 0.0034. The combined series give a straight line for which μ = 12,500 calories. This represents the mean for the range 10–38°, and is subject to a probable error of ±0.6 per cent. The vertical width of the band of points, which as in other cases (Crozier and Stier, 1924–25) forms a ribbon with parallel margins, is an index of the variability encountered in the normal cockroach. On the basis of a selection of sets of readings representing wider deviations than usual in the data here reported, the latitude of variation for any one individual is with very few exceptions well within ±7 per cent of the mean frequency at any one temperature. On a like basis, the probable error of the mean frequencies entered in the graphs is found seldom to exceed ±2 per cent, being much less in about half the observations.

In contrast to the others, one individual yielded data from which a clearly different magnitude of μ must be derived. When a first "run" revealed a value of the order of 14,300, I made additional series of observations upon the same individual within 18 days after the first. Considered separately, the latter series give rise to values of μ respectively higher and lower than the initial determination, yet safely

[3] A very slightly lower average value is given by the "runs" of falling temperatures than by those of rising temperatures, but the difference is not great or consistent enough to be significant. Thus, of six comparable series (Fig. 1) the three during which the temperature was lowered give 12,020, 12,210, and 12,500 (mean = 12,240), and the other three during which the temperature was raised give 12,060, 12,670, and 12,810 (mean = 12,510).

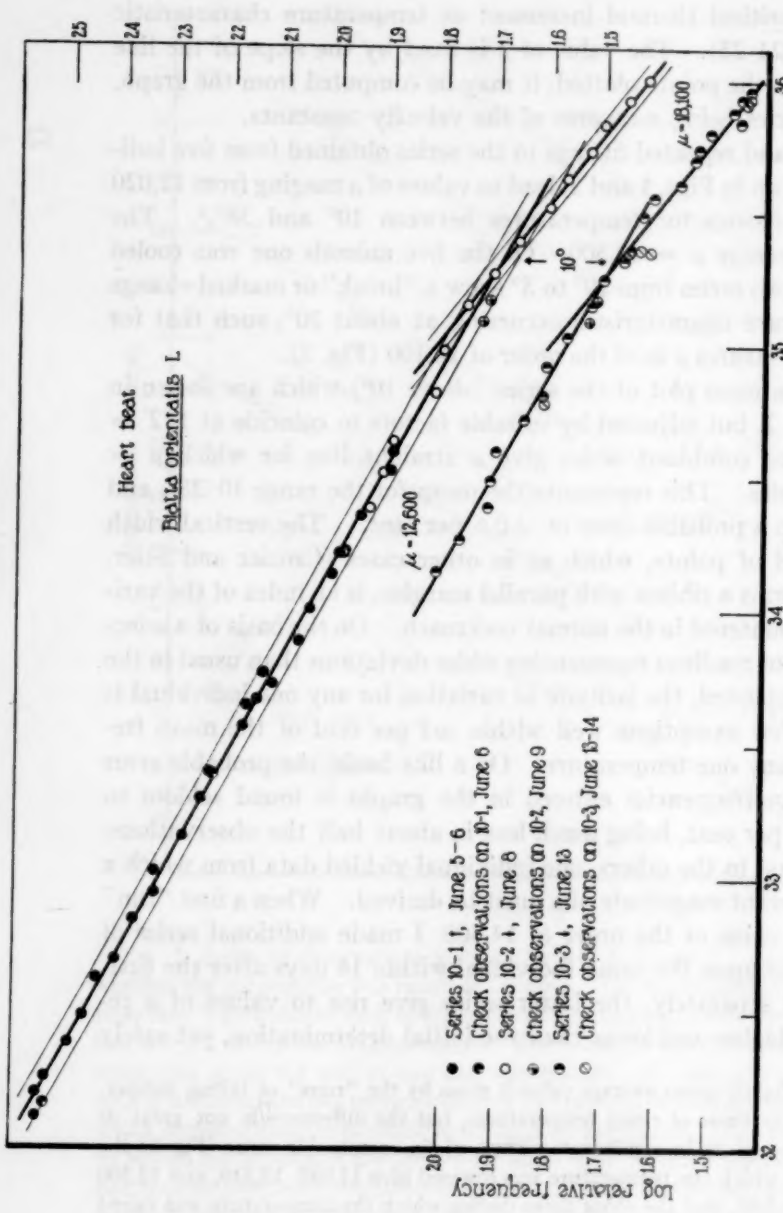

FIG. 2. Series of observations obtained with Animal 10. Separation of two "runs" is effected by plotting against ordinates at left and right, the scales differing by 0.2 of log frequency. The final (lower) series would otherwise fall only about 0.007 unit below the combined upper series. The direction of change of temperature in each series of readings is indicated by the arrows. (Since check observations on returns from extreme low temperatures revealed a lag in resumption of higher frequency, such points were not accorded full weight in fitting the lines.) It is to be observed that if one were in possession merely of data from a series of readings over the temperature range 5–19°, difficulty would be experienced in obtaining a significant μ, and the data as plotted might seem to fall upon a curve.

by the arrows. (Since check observations on returns from extreme low temperatures revealed a lag in resumption of higher frequency, such points were not accorded full weight in fitting the lines.) It is to be observed that if one were in possession merely of data from a series of readings over the temperature range 5–19°, difficulty would be experienced in obtaining a significant μ, and the data as plotted might seem to fall upon a curve.

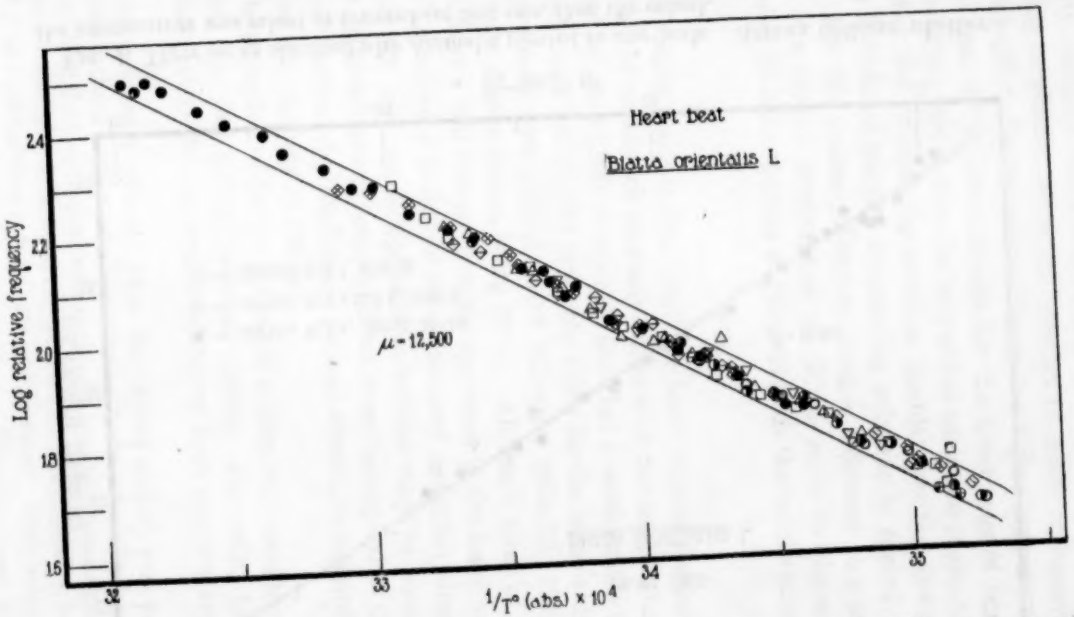

FIG. 3. Combination of series (above 10°) obtained with Animals 4, 5, 6, 7, and 10, all series being made to coincide (by suitable factors) at $1/T = 0.0034$. The symbols are the same as in Figs. 1 and 2.

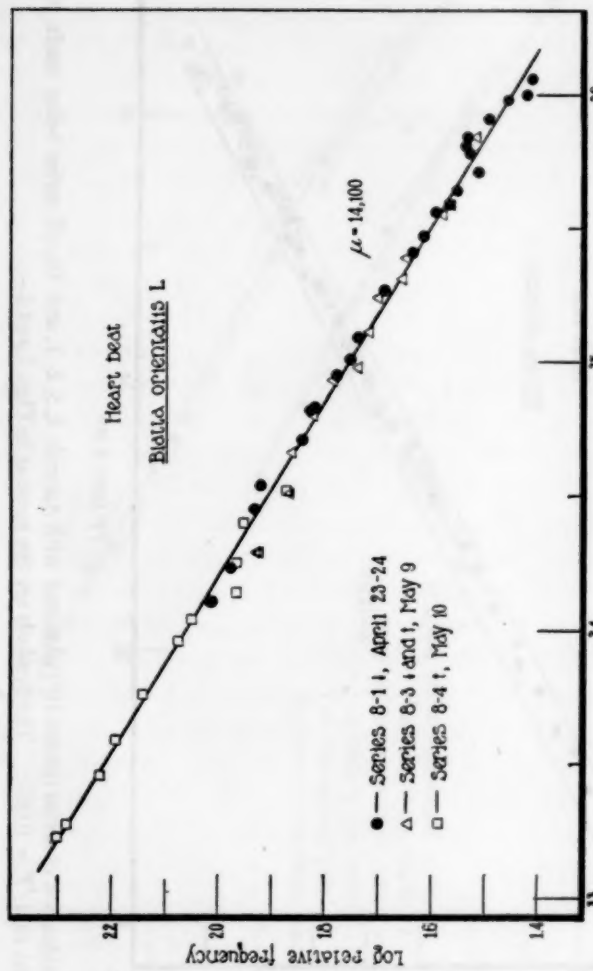

FIG. 4. Three series obtained with Animal 8 plotted to one scale. Arrows indicate whether the temperature was raised or lowered (or first one, then the other).

comparable and not to be classed with the previous cases averaging 12,500. Fig. 4 shows the data from three "runs." Coincidence is so nearly complete that a single line is drawn to represent the average, according to which μ = 14,100 (correct to ±300 or better) over the range 4.5–28°.[4] In this case no change to a higher temperature characteristic near 10° is demonstrable for the lower rage of temperature. Excepting the first series, the latitude of variation is greater than for the other five animals (as much as double), yet the probable errors of the individual plotted frequencies only rarely exceed ± 2.5 per cent. For this animal, therefore, both a difference in μ and the absence of a critical temperature at 10° ± serve to characterize the control of its heart rhythm as different from that of the others.

III.

The results make it clear that the heart rate in the late nymphal stage of the cockroach typically varies with the temperature in a way defined by μ = 12,500 ± calories; but that a considerably higher critical increment (*ca.* 18,100) probably holds for the same animals at low temperatures, the critical point (Crozier, 1924–25, 1925–26, *a*) at which the change occurs being 10°±. Although most of the animals reveal above this temperature an approach to the mean value 12,500, with a constancy indicated by a standard deviation amounting only to 2.3 per cent, an exceptional individual may be found which in some (still unknown) respect differs from the typical so that the control of the heart rhythm lies in a process whose μ is definitely of another magnitude, namely 14,100±.

The pulsation of the dorsal vessel of insects is considered to be controlled by the central nervous system (Zawarzin, 1910–11), and Carlson (1905–06) reported evidence of both augmentary and inhibi-

[4] Another series obtained from this animal showed such abnormally high variability that μ could not be determined with any reasonable degree of accuracy. Except that the observations were in this instance begun immediately after transference to the tube, little cause can be given for this instability, but a correlation with it of a general drop in heart rate may be noted. Both occur in cases of subnormal vitality, as other observations show. Thus Cockroach 10 showed both a marked increase in latitude of variation and an absolute slowing down of the heart rhythm after 4 weeks in the experimental tube.

tory innervation of the heart of an orthopteran (*Dictyphorus*).[5] The typical value of μ (12,500) for the cockroach heart rate was therefore expected to accord quantitatively with values determined for other non-respiratory neuromuscular activities of arthropods presumed to depend upon the rate of "central nervous discharge" (Crozier, 1924–25). It does so within limits of difference ascribable to errors of curve fitting or to consequences of uncontrolled body or limb movements. Thus Crozier and Stier (1924–25) have listed a number of such phenomena, for which $\mu = 12{,}200\pm$. More recently they have (1925–26) reported the same value applying to locomotor activity in tent caterpillars. In addition, Federighi[6] finds the heart beat of the annelid *Nereis* to show $\mu = 12{,}400$.

Neither is the exceptional case where $\mu = 14{,}100\pm$ entirely without counterpart, although it is very infrequent (Crozier, 1925–26, *b*). A like value appeared (as an exception in series yielding 16,200) among Glaser's (1925–26) determinations for the heart rate of a pteropod and (as exception to 11,100) in Cole's (1925–26) findings for locomotion in *Planaria*. The data in the present instance offer nothing toward explaining the atypical value, for no difference was apparent in the treatment or condition of the animals. All that can be said is that the results point to the possible validity of 14,000+ as the temperature characteristic of one of several chemical reactions which may be supposed necessary to more than one type of vital process (Crozier, 1925–26 *a*, *b*) but which only exceptionally proceeds so slowly as to assume a governing rôle.

IV.

SUMMARY.

The frequency of pulsation of the intact heart in nymphs (final (?) instar) of *Blatta orientalis* L. increases with the temperature according to the equation of Arrhenius. The constant μ has typically the same value, within reasonable limits of error, as that (12,200) deduced for other, homologous activities of arthropods where the

[5] More recently Alexandrowicz (1926) has described in detail the innervation of the cockroach heart.

[6] Federighi, H. (unpublished experiments).

rate of central nervous discharge is perhaps the controlling element, namely 12,500 ± calories for temperatures 10–38°C. Below a critical temperature of about 10° a change to a higher value of the temperature characteristic occurs, such that μ = 18,100 ±. Exceptionally (one individual) μ = 14,100 ± over the whole range of observed temperature (4.5–28°).

The quantitative correspondence of μ for frequency of heart beat in different arthropods adds weight to the conception that this constant may be employed for the recognition of controlling processes.

It is a pleasure to acknowledge my indebtedness to Professor W. J. Crozier for his suggestion and guidance of this work.

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SOME PHYSICOCHEMICAL PROPERTIES OF DISSOCIATED SPONGE CELLS.

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I. INTRODUCTION.

It has been known since Wilson's discovery (1) that dissociated cells of *Microciona* come together and form aggregates, which by further transformation develop into new sponges. Similar processes were observed in fresh water and in calcareous sponges (2, 3), and in hydroids and alcyonarians (4, 5). Recently one of us (6, 7), in studying the behavior of dissociated cells of *Microciona*, found that the formation of aggregates is due to the ameboid movement of so called archæocytes, that is, unspecialized cells of the sponge mesenchyme, which upon separation creep in various directions and coalesce with other cells of the same species which happen to lie on their route. According to these observations, aggregation is easily affected by changes in the surrounding medium. In pure isotonic solutions of NaCl or KCl the ameboid movement is entirely inhibited, and the addition of at least one of the alkaline earth metals, either Ca or Mg, is necessary to produce the aggregation of cells. The addition of acids or bases to a suspension of cells also causes significant changes in their behavior, inhibiting their movement and changing the adhesive properties of the protoplasm. In mixed suspension, the cells coalesce only with cells of their own species, forming separate aggregates; while in alkaline sea water the *Microciona* aggregates become surrounded by the *Cliona* cells.

The present investigation is an attempt to deal in a quantitative manner with the equilibrium relations involved between the cells of two siliceous sponges, *Microciona prolifera* Ver. and *Cliona celata* Gr., and acid or base.

As a preliminary to the account of the investigation of the sponge cells, we report a titration of 0.00280 molar NaAc in 0.520 molar NaCl solution; this solution served as the medium in experiments with the cells.

II. The Hydrogen Ion Activity in a Solution Containing 0.00280 Mols of NaAc, 0.520 mols of NaCl per Liter, and Various Amounts of HCl and NaOH.

A medium for the titration of the cells of the sponges must answer several requirements. First of all, it must be isotonic with the cells; secondly, it must be of such a nature as to prevent aggregation of the cells (7); and, thirdly, it must have a certain buffer value on the acid side of neutrality (since most of the observations were made in that range) so as to yield reproducible E. M. F. measurements.

After several trials we found that a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl, and varied amounts of HCl and NaOH, answered practically all of our requirements. It gives fairly reproducible E.M.F. measurements, but its buffer value is not large enough to mask the effect of the acid or base bound by the sponge cells.

Several investigations were made on acetate buffers containing different amounts of NaCl. L. Michaelis and R. Krüger (8) studied the hydrogen ion activity of a 0.02 N mixture of equal amounts of NaAc and HAc in the different salts. They found that in 1 molar NaCl the mixture has a pH of 4.484, the pK′ evidently being equal to 4.484.

L. Michaelis and K. Kakinuma (9) in their contribution to the electrochemical measurements of the activity of ions found that 0.01 molar solutions of equal amounts of NaAc and HAc, containing different amounts of NaCl, have different hydrogen ion activities. A solution containing 0.1 mol of NaCl had a pH equal to 4.607, a 0.5 molar solution a pH of 4.503, and a 1.0 molar solution a pH of 4.448.

G. S. Walpole (10) investigated the pH of a mixture of HAc, NaAc, and NaCl. The concentration of Ac in this system was 0.20 N; a 1:1 mixture gave a pH of 4.58. The measurements were made at 18°C.

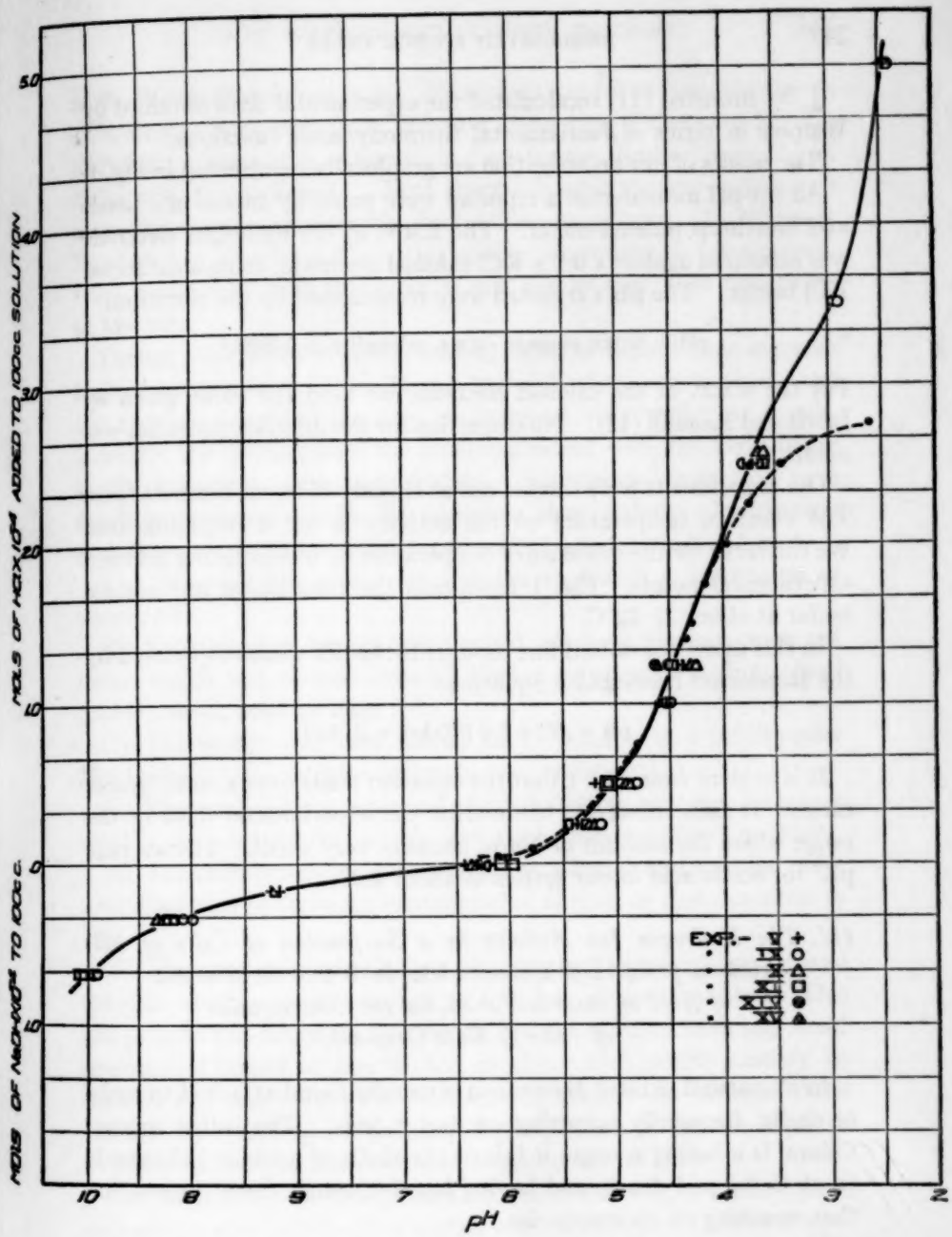

FIG. 1. The hydrogen ion activity in a solution containing 0.00280 mols NaAc, 0.520 mols NaCl per liter, and varied amounts of HCl or NaOH.

J. N. Brönsted (11) recalculated the experimental data obtained by Walpole in terms of fundamental thermodynamic functions.

The results of our investigation are graphically represented in Fig. 1.

All the pH measurements reported were made by means of a Leeds and Northrup potentiometer. The E.M.F. of the hydrogen electrode was measured against a 0.1 N KCl calomel electrode, using a saturated KCl bridge. The pH's reported were recalculated by the equation:

$$\text{pH} = (\text{E.M.F. observed} - \text{E.M.F. calomel})\ 1 \div 0.001983$$

For the E.M.F. of the calomel electrode we used the value given by Lewis and Randall (12). No correction for the diffusion potential was made.

Our experiments were carried out at slightly different temperatures. The effect of temperature on the activity is not a negligible one. We corrected for the influence of temperature by interpolating between experimental points. Fig. 1 represents the titration of our acetate buffer at about 21–22°C.

In this figure the dotted line represents the pH values calculated by the Henderson-Hasselbalch equation:

$$\text{pH} = \text{pK}' + \log [(\text{NaAc}) \div (\text{HAc})]$$

It is evident from Fig. 1 that the equation holds over a considerable range. It fails, however, to describe the experimental data in the range where the amount of NaAc becomes very small. The average pK′ for acetic acid in our system is about 4.37.

III. The Hydrogen Ion Activity in a Suspension of Cells of Microciona prolifera or Cliona celata, in Which the Amount of Acid or Base is Varied, but the Concentration of Cells Is Kept Constant.

In its natural habitat *Microciona* is usually found attached to rocks or shells, frequently occurring on oyster beds. The sulfur sponge, *Cliona*, is a boring sponge; it infests the shells of various pelecypods (both living and dead), and having bored through them, grows farther, reaching an enormous size.

The sponges used in the experiments were collected in the vicinity

of the Woods Hole laboratory. *Microciona* was taken from the mouth of Wareham Bay, and from Waquoit Pond near Falmouth, Massachusetts. *Cliona* was obtained in Squeteague Harbor near North Falmouth, Massachusetts. Both species inhabit shallow waters and normally sustain considerable fluctuations in salinity. In Waquoit Pond and in Wareham River the salinity, according to our observations, varies with each tide from 27 gm. per liter at high tide to 16 at low tide.

Though the salinity in the laboratory tanks at Woods Hole is much higher, varying from 31 to 32, no harmful effect was noticed, and the sponges sustained the new environment very well. As a rule, however, the sponges used for experimentation were not kept longer in the aquarium tanks than 5 days. Experience shows that as a result of prolonged life in the aquarium they undergo physiological and anatomical changes, and become unfit for experimental work.

The following procedure was adopted to obtain a suspension of sponge cells.

(1) Each piece of sponge was washed, all dead portions were cut away, and it was cleaned from all foreign substances, such as small pebbles, sand, mud, or algæ.

(2) The sponge was then placed for 15 minutes in a 0.520 molar NaCl solution, the solution being changed twice.

(3) The material was squeezed through bolting silk No. 20 into a solution containing 0.00280 mols NaAc and 0.520 mols NaCl per liter.

(4) The next procedure consisted in centrifuging and washing twice with a solution of the same concentration of salts as that described in (3).

(5) The suspension of cells thus obtained was transferred to a vessel through which a steady current of CO_2-free air was bubbled. This last process was necessary in order to free the solution from any small amounts of bound or free carbon dioxide which might possibly be present, and also to keep the cells from settling to the bottom of the vessel. By bubbling air through the suspension, it can be kept for 24 hours without sedimentation and aggregation of cells.

The suspension of sponge cells obtained consists of archæocytes, collencytes, pinacocytes, desmacytes, and choanocytes. The percentage composition of the suspension may be given as follows: *Micro-*

ciona; 25.5 per cent archæocytes, 9.9 per cent collencytes, and 64.6 per cent desmacytes, pinacocytes, and choanocytes; *Cliona*; 15.4 per cent archæocytes, 15.0 per cent collencytes, 69.0 per cent desmacytes, pinacocytes, and choanocytes.

During the preparation of the suspension the cells were subjected to rather vigorous mechanical treatment. Part of them might have been cytolyzed. The products of this cytolysis might appear in the watery phase, and might be responsible for the binding of any acid or base added to the system.

TABLE I.

Effect of the Number of Washings on the pH of a Suspension of Cells of Microciona prolifera.

Experiment VI. Washing solution contains 0.520 mols of NaCl, 0.00280 mols of NaAc, and 1.25×10^{-3} mols of HCl per liter. 30 minute intervals between consecutive washings.

No. of washings. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
	volts	°C.	
2	0.6230	23.8	4.88
2	0.6218	23.8	4.86
3	0.6274	23.8	4.95
3	0.6272	23.8	4.95
4	0.6251	24.2	4.91
4	0.6247	24.3	4.90
5	0.6235	24.2	4.88
5	0.6233	24.2	4.88
6	0.6205	24.1	4.83
6	0.6206	24.1	4.83
NaCl solution used in this experiment.	0.5952	24.7	4.40
	0.5960	24.7	4.41

In order to determine whether we were dealing in our experiments with the acid- or base-binding property of the cells, or with the effect of some unknown product of cytolysis, we carried out the experiment reported in Table I. In this experiment a suspension, prepared in the way already described, was further washed with a solution containing 0.00280 mols of NaAc and 0.520 mols of NaCl per liter. After each washing the suspension was centrifuged, and the pH of the super-

natant liquid determined. It is evident that the pH of the suspension remains practically constant. If the acid-binding property of this system was dependent upon the product of cytolysis, the pH should gradually have risen until it reached the pH of the washing solution. No such phenomenon was observed. We must conclude, therefore, that in this case we are dealing with a property very closely associated with the living cells.

In our experiment we added acid or base to the suspension of the sponge cells. When any acid or base is added to a system containing

TABLE II.

Effect of Time upon the Establishment of an Equilibrium between the Cells and the Acid Added.

Solution: 0.520 mols NaCl and 0.00280 mols NaAc per liter.

Suspension: 51.9×10^8 cells of *Microciona* suspended in 100 cc. of solution; 1.25×10^{-4} mols HCl added to it.

Time elapsing between the addition of the acid and the E.M.F. measurement. (1)	E.M.F. (2)	Temperature. (3)	pH (4)
min.	*volts*	°C.	
48	0.6219	21.8	4.892
	0.6218	21.8	4.890
63	0.6223	22.0	4.896
	0 6223	22.0	4.896
99	0.6241	22.0	4.919
	0.6240	22.0	4.918
125	0.6249	22.4	4.933
	0.6248	22.4	4.931

basic or acid radicals, a displacement of the equilibrium occurs. The establishment of the new equilibrium takes a certain length of time, depending upon the properties of the system. To test the effect of time on the system containing a rather large amount of acid and a suspension of cells of *Microciona prolifera*, we carried out the experiment reported in Table II. As is seen from the table, the pH values are almost constant. The difference in the pH value of the cell suspen-

sion and the same solution without the cells, in this experiment, is equal to 0.5 of a pH unit, while the difference between the first reading and the last is only 0.04 of a pH unit. There are three possible

TABLE III.

Vitality Tests of the Cells Used in the Titration Experiments.
Microciona prolifera.

Experiment No.	pH of the suspension.	Condition of cells after titration experiments.	
		Immediately.	12 hrs. later.
(1)	(2)	(3)	(4)
VIII	4.56	Normal.	Very small aggregates slightly adhering to glass; many cytolyzed cells.
V	4.82	"	Small globular aggregates adhering to glass; few cytolyzed cells.
III	5.90	"	Normal aggregates adhering to glass; few cytolyzed cells.
V	6.13	"	Normal aggregates adhering to glass.
X	6.36	"	" " " " "
X	6.96	"	" " " " "

TABLE IV.

Vitality Tests of the Cells Used in the Titration Experiments.
Cliona celata.

Experiment No.	pH of the suspension.	Condition of cells after titration experiments.	
		Immediately.	12 hrs. later.
(1)	(2)	(3)	(4)
IX	3.06	Part of cells cytolyzed.	Cells strongly adhering to glass; no aggregation.
IX	3.71	Normal.	" " " " " " "
IX	4.09	"	" " " " " " "
IX	6.59	"	Normal aggregates.
IX	6.70	"	" "
XI	7.34	"	" "

causes of this variation. One of them has already been pointed out; namely, the time factor in the establishment of the new equilibrium. The second factor which must undoubtedly be present in any system containing living material is that of metabolism. The products of

the metabolism might possess acid or basic properties of their own, and might gradually change the pH of the medium. Without knowing the chemical nature of these metabolic products, one cannot determine their influence upon the pH of the suspension. The third factor which may be responsible for this variation is the beginning of cytolysis. It is quite probable that in such acid solution irreversible changes occur in the cells, producing more and more titratable material. This will be seen from the discussion of the vitality tests of the cells treated with acid.

In all subsequent measurements we used 45 minutes for the equilibration time.

The next problem with which an investigator of living matter is confronted, is to determine whether or not his chemical manipulations have caused a permanent injury or death to the object of his experiment. The best test of this, is to examine the cells immediately after the experiment and to observe their behavior when they are brought back to a normal environment.

For this purpose we conducted the following tests in conjunction with the measurements of the pH which resulted from the addition of acid or base to the suspension of sponge cells: the cells used in the experiments were washed with sea water and examined under the microscope to determine whether they were alive or not, then 1 cc. of suspension was added to 9 cc. of sea water, and the mixture left undisturbed for 12 hours. This period is long enough for uninjured cells to coalesce and form globular aggregates which adhere strongly to the glass (7). The cells irreversibly affected by previous treatment are partially cytolyzed and form aggregates of irregular shape. Dead cells form loose sediment not adhering to the glass.

Tables III and IV list the results of the vitality tests carried out on cells taken from the titration experiments. It can easily be seen that the susceptibility of *Microciona prolifera* to acid solutions used in the experiments is much higher than that of *Cliona celata*. Though at the end of Experiments V and VIII the cells of the former remain alive and under the microscope appear to be normal, their ameboid movement is retarded. After 12 hours, instead of forming a few large aggregates as always happens under normal conditions, they coalesce into numerous small groups; many cells at the end of this period be-

come cytolyzed. This occurs when the pH of the supernatant liquid of the suspension is 4.56.

The cells of *Cliona celata* endure much higher acidity remaining uninjured at pH 3.71, though their ameboid movement after such treatment is inhibited.

The difference between the cells of the two sponges can undoubtedly be correlated with the fact that for a given change in pH in fairly acid solutions, *Microciona* binds more acid than *Cliona*. We may suspect therefore that greater chemical changes occur in the first than in the second.

It must be borne in mind that, due to the rough treatment during squeezing, washing, and centrifuging, the cell suspensions may contain a certain amount of cytolyzed material; so the presence of a small number of cytolyzed cells cannot be attributed entirely to the effect of acid solution. Further increase in hydrogen ion activity will certainly cause a complete cytolysis and death of the cells. As can be concluded from the examination of Tables III and IV, the critical concentration probably lies just below pH 4.5 for *Microciona* and about 3.7 for *Cliona*. Above these values, the largest part of the cells examined under the microscope immediately after the titration experiments showed no evidence of injury.

The amount of acid or base bound in titration of a suspension depends upon the concentration of cells in that suspension. Our titration experiments were carried out with suspensions of a definite concentration. We shall express this concentration in terms of the number of cells present in 100 cc. of the suspension. This method of expressing the concentration is not strictly correct. Cells have their own volume; therefore the volume of "free" solution depends upon the number of cells present. Any computation of acid or base bound referred to 100 cc. will deviate from the true value by the volume of cells present in the system. However, we believe that by using rather dilute suspensions of cells we made this error negligibly small.

In determining the number of cells in a given suspension, the following procedure was adopted: 1 cc. of this suspension was diluted one hundred times and shaken well. 1 cc. of this suspension was transferred to a counting cell. A uniform distribution of the cells was secured by the use of a pipette. In about 10 minutes the cells settled

on the bottom and could be counted with a Whipple square micrometer. We counted the cells in ten fields of view taken at random at various parts of the counting cell. From the average number obtained by this procedure the total number of cells in 1.00 cc. of suspension was estimated. The results were usually accurate within 5 per cent.

If the stock suspension was found to differ from the desired concentration, it was diluted to the appropriate extent. After the dilution, the number of cells was checked once more.

In all our experiments we titrated with HCl and NaOH, and for this reason our medium contained a large amount of NaCl. Any addition of small quantities of Cl or Na produced, therefore, a practically negligible change in the concentration of either Na or Cl. Any reaction of the cells will, therefore, be due entirely to the change in the concentration of free acid or base as measured by the hydrogen ion activity or its dependent variable, the hydroxye ion activity. This statement would be accurate if our systems had not contained NaAc. Upon the addition of an acid, however, the concentration of Ac^- decreases proportionally to the acid added. Therefore, in addition to the variables (H^+) and (OH^-) we have the variables (HAc) and (Ac^-). Evidently this second set of variables cannot be neglected. A simple way to test the influence, if any, of the concentration of HAc and Ac^- is to titrate the cells in a solution of 0.520 molar NaCl in the absence of NaAc. Such an experiment is hardly quantitative in a slightly acid solution, but in a medium containing a rather large amount of acid the E.M.F. becomes reliable. Therefore we brought a solution containing 0.520 mols of NaCl per liter to a pH of 4.50 by adding to it a known amount of HCl. Then to the same solution we added about 50×10^6 cells of *Microciona prolifera* and by the addition of HCl brought it to the same pH as the solution of NaCl. It was found necessary to add more acid to the cells than to the NaCl solution in order to make the two solutions isohydrionic. Evidently the amount of acid added to the cells minus the amount of acid added to the NaCl solution is the amount of acid bound by the cells. It was found to be equal to 1.2 ± 0.2 mols HCl $\times 10^{-4}$. If we compare this figure with the one obtained from the titration of the cells of *Microciona prolifera* in the presence of NaAc (Fig. 2) we find a complete agreement. Evidently the (HAc) and (Ac^-) are not the controlling factors in the titration in question.

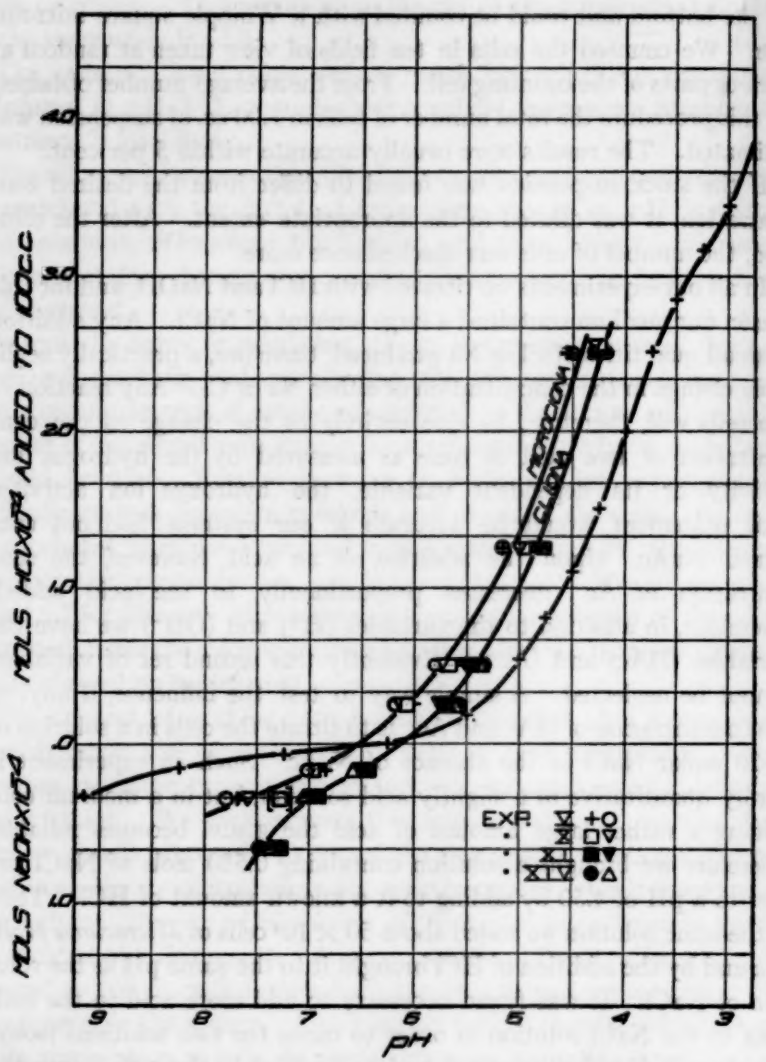

FIG. 2. The hydrogen ion activity in a solution containing NaAc and NaCl, a given number of cells of *Microciona prolifera* or *Cliona celata*, and varied amounts of HCl or NaOH.

Microciona: Experiment V, 52.4 × 10^8 cells; Experiment X, 49.3 × 10^8 cells. *Cliona:* Experiment XI, 57 × 10^8 cells; Experiment XIV, 51.1 × 10^8 cells.

The titration of the suspension was effected by adding to a known amount of cells a given amount of HCl or NaOH. The cells were then centrifuged and the pH determined electrometrically on the supernatant liquid. Two E.M.F. measurements were carried out on each sample. The results of two experiments are given in Fig. 2, together with the titration of the acetate buffer, taken from Fig. 1.

As may be noticed from the titration curve of *Microciona prolifera*, the two experiments disagree slightly with each other in the upper part of the curve. The reason for this disagreement is a difference in concentration of the cells in the two experiments. By drawing a line between the experimental points we can take care of this influence, and the line of titration would represent a titration of suspension having approximately 50.8×10^8 cells per 100 cc. A similar behavior is shown by the cells of *Cliona*, though to a lesser extent.

The titration curves obtained for the sponge cells are only functions related to the acid- or base-binding properties of the cells. The curves will have different shapes in media containing different buffers.

If we subtract at any pH the amount of acid necessary to bring the acetate buffer to that pH from the amount of acid added to the cells to bring them to the same pH, we shall obtain a value characteristic of the suspension—the amount bound by the suspension.[1]

Such a calculation was made for both *Microciona* and *Cliona* for the slightly acid and basic ranges of the titration curve. The results of the estimates are given in Fig. 3. They are probably accurate within about 8 per cent.

The function thus obtained is of fundamental importance for the estimation of the physicochemical properties of cells. Each curve has

[1] This procedure is not strictly correct since the free base or acid is related to the hydrogen ion activity by the equations:

$$(HCl) = \gamma_1 (a_{H^+})$$

$$(NaOH) = \gamma_2 K_w \div (a_{H^+})$$

in which the activity coefficients γ_1 and γ_2 vary with the change in concentration of HCl and NaOH.

But, since our system contains a large amount of NaCl, the change in the activity coefficients between the acetate titration curve and the one of the sponge cells is probably small.

two parts; one above the zero point where the sponge behaves as a base, and one below where it behaves as an acid. The zero point,

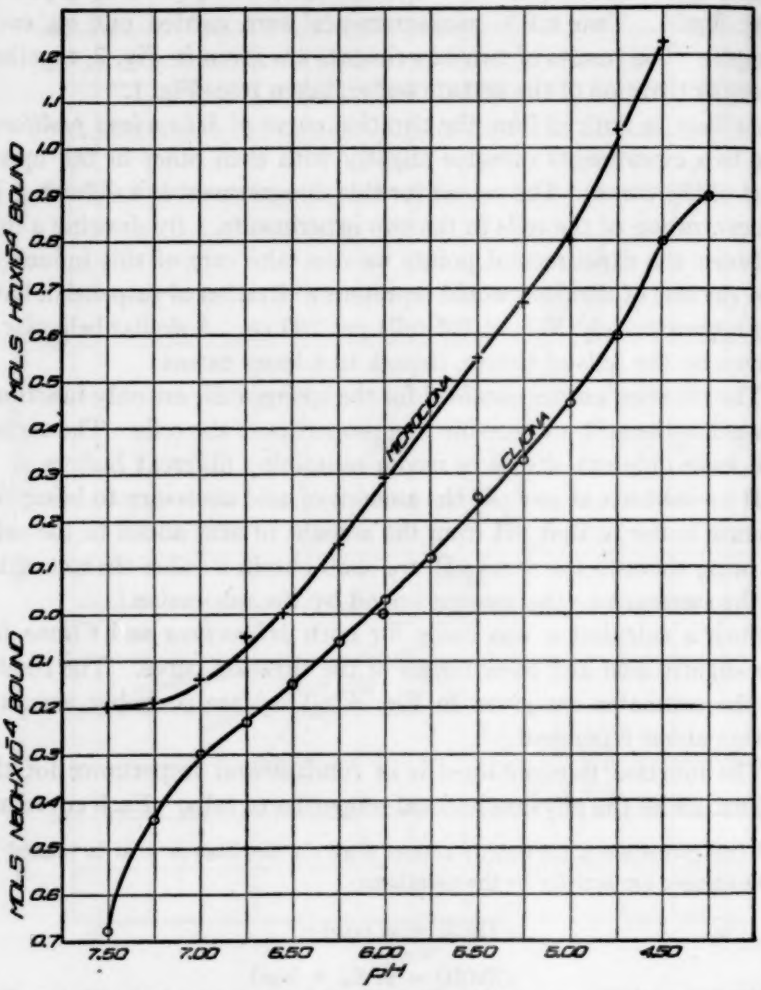

FIG. 3. The acid and base bound by the cells of *Microciona prolifera* and *Cliona celata* at different hydrogen ion activities.

where no base or acid is bound, is of considerable interest for us. It represents the pH of a pure suspension of cells, extracted from the

sponge and washed with isotonic NaCl. These pH values for extracted cells are different for *Microciona* and *Cliona*. The cells of *Cliona* are more acidic than those of *Microciona*. We believe that these pH values may be characteristic of the species concerned, provided the comparison is made at a concentration of about 50–60 $\times$ 10^8 cells.

The hydrogen ion activity of the original suspension for *Microciona prolifera* is equal to a pH value of 6.55 $\pm$ 0.1, and for *Cliona celata* to a pH value of 6.10 $\pm$ 0.1.

Passing to the acid range of the acid- or base-binding curve we observe that *Cliona*, being more acidic than *Microciona*, behaves as a weaker base, and binds less acid than the latter. *Microciona*, being more basic, binds more acid for a given change in pH.

The basic part of the acid- or base-binding curve is even more characteristic for the two species of sponges. While *Microciona prolifera* is almost saturated with the small amount of base at a pH value of 7.50, *Cliona* still has a considerable base-binding capacity at that point. It substantiates once more the conclusion reached upon comparison of the two species in the acid portion of the curve; namely, that *Cliona* behaves as a much stronger acid than *Microciona*.

This conclusion, however, is open to one criticism: the suspensions of the cells of *Microciona* and *Cliona*, though containing an equal number of cells, are composed of cells of different sizes. Therefore, the total surface of the cells is different for *Microciona* and *Cliona*.

If the removal of acid or base from the liquid phase, by the cells, is entirely due to the effect of the active surface, the results reached in this investigation would seem to refer to the surface, but not to the chemical properties of the body of the cells.

It is therefore of considerable interest to provide an experimental evidence to prove that the reagents used penetrated inside the cells.

In the course of the investigation upon the cells of *Cliona* it was found that these cells changed their coloration from yellow to dark brown at a pH ranging from 7.3 to 7.4. Upon microscopic examination of the cells, it was observed that the yellow pigmented granules of the cells were responsible for this change in color.

On treating the cells with absolute ethyl alcohol, this pigment can be extracted, and the same change in color reproduced in a test-tube.

These experiments indicate that the cells of *Cliona* in faintly alka-

line solution are permeable to our reagents. The reaction is not limited to the surface of the cell.

This evidence cannot, however, be extended to the acid range of titration of *Cliona*, nor to *Microciona* suspensions; but, since we have no reasons for believing that an entirely different physicochemical mechanism is involved in these cases, we are inclined to think that the action of our reagents is not limited to the surfaces of the cells.

We may conclude, therefore, that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

IV. CONCLUSIONS.

1. The activity of the hydrogen ion, in a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl per liter, and varied amounts of HCl or NaOH has been investigated. The average value of pK′ for acetic acid in this system is about 4.37.

2. The effect of the addition of various amounts of HCl and NaOH to a system containing 0.00280 mols of NaAc, 0.520 mols of NaCl, and a known number of cells of either *Microciona prolifera* or *Cliona celata* was then studied. It was found that in weak acid solutions *Microciona* behaves as a stronger base than *Cliona*, the former being practically saturated with base at a pH of 7.5. Similar behavior is shown by suspensions of cells to which no acid or base was added: the cells of *Cliona* are more acidic than the cells of *Microciona*.

3. The microscopic examinations of the cells subjected to the treatment with acid or base indicate that the cells of *Microciona* remain alive down to pH 4.50; the cells of *Cliona* sustain greater acidity,—at pH 3.7 they exhibit no signs of cytolysis. Tests for aggregation of these cells showed that this phenomenon is greatly inhibited even by slightly acid solutions.

4. The conclusion is drawn that the concentration of cells being equal, the suspensions of cells of *Microciona* and *Cliona* differ from each other in their physicochemical properties, the comparison being made on suspensions of specified composition.

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THE GEOTROPIC CONDUCT OF YOUNG RATS.

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I.

If tropistic behavior is to be utilized for ultimate analysis of the inner processes controlling conduct it is quite necessary that the most complete possible mathematical expressions be found for at least several different modes of response. Only in the case of phototropism has any considerable progress in this direction been achieved. We have reference, not so much to the theory of sensory activation, as to the reasonably complete formulation of relationships between the magnitude of the excitatory intensity and the speed and extent of the induced orientation. In this respect the knowledge of geotropism, by contrast, is singularly defective.

For plants, it is found that geotropic excitation is proportional to the sine of the angle of the stimulated part with the horizontal (Fitting, 1905; Pekelharing, 1910). From the relationship between mass of attached leaf and rate of geotropic curvature in horizontal stems of *Bryophyllum*, Loeb (1918, 1924) inferred that the curvature was dependent upon the amount of (gravitationally directed) substance sent into the stem by the leaf. This is obviously consistent with the finding that the "presentation time" for geotropic response is directly proportional to the effective gravitational component (Pekelharing, 1910).

Quite recently the question of geotropic orientation in animals has been reexamined by Cole (1925–26), from the standpoint of the rôle taken by direct action of gravity as leading to tensions produced in muscles which support the organism's weight. With *Helix* Cole was able to show that the speed of upward creeping, after orientation is accomplished, increases with the sine of the angle of inclination of the creeping surface, and thus as the active component of gravity. This leads to the view, substantiated by the effects of forcing such an

animal to carry additional loads (Crozier and Federighi, 1924–25; Cole, 1925–26), that orientation is controlled not by some statocyst function but by the differential gravitational pull upon the two sides of the body (Loeb, 1897),—a view earlier advocated for Chitons (Arey and Crozier, 1919), which lack the statocyst of gasteropods.

The information we desired to obtain for the analytical account of geotropism required data upon the amount of upward orientation in a negatively geotropic animal, and the precision of this orientation, as related to the inclination of the creeping surface. For reasons indicated in earlier papers (Crozier and Pincus, 1926–27, *a*, *b;* 1926) we have employed for these experiments young rats of known genetic history, studied during the period of about 2 days which intervenes between the 12th day after birth and the time when the eyelids opened. It happens that with these animals certain new or hitherto ignored features of the geotropic response become apparent and greatly improve the opportunities for investigation. The result seems to indicate quite clearly a direct dependence of orientation upon the distribution of the animal's weight upon the legs of the two sides of the body. Formulæ are derivable describing the orientation with considerable exactness.

We regard it as an interesting fact that, for the first time, a detailed account of a tropism is possible which is based upon experiments with a mammal. This amounts to a sort of reversal of anthropomorphism, and constitutes a decided obstacle for those who would emphasize the greater "simplicity" of lower animals. The simplicity of conduct which permits the mathematical formulation of a mode of behavior is not so much a matter of zoological affinity as it is of dynamical symmetry in the organism and of the choice of experimental conditions which permit the animal to display its potentialities as a machine.

II.

In order to record trails of geotropic orientation each rat was placed upon a fine-meshed wire grid, which permitted a good foothold for creeping. When placed on the creeping plane the axis of the body was at first horizontal, or, occasionally, with the head pointing downward. The tilt of the wire surface was measured on a protractor.

The angle θ was measured when the animal had oriented and was creeping steadily. The correspondence of the wire grid to coordinate paper made it possible to copy the path upon record sheets. The path was indicated either by marking with chalk the position of the rat's axis on the wire, or by placing a straight-edge parallel to the axis. The central stripe due to the hooding factor facilitated such procedure. The path of orientation is a straight line, as shown diagrammatically in Fig. 1, unless, after "hesitation," the rat veers to the opposite side—in which case the angle θ is found to be the same.

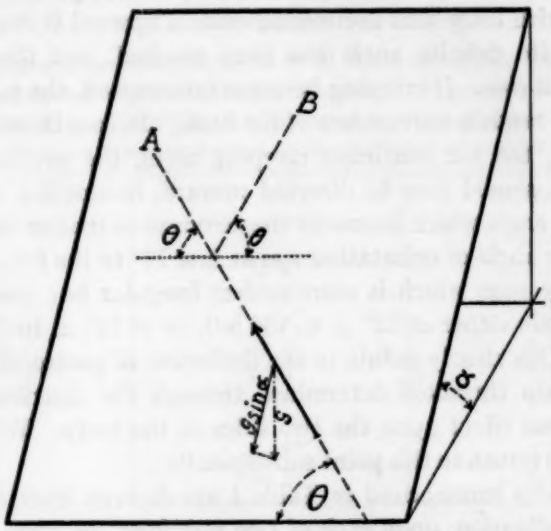

FIG. 1. Diagram showing terms used in description of orientation of rats creeping upon a wire grid inclined at α to the horizontal. The position of orientation is defined by the angle θ, the active component of gravity being $G \sin \alpha$. As described in the text, the Path A may be steadily pursued, or the animal may swing to one (B) equally inclined but in the opposite direction.

To obtain data which might be legititimately averaged it is necessary to employ rats as closely comparable as possible. Two albino rats, aged 13 to 14 days, were used in obtaining the records in Fig. 7. Those employed for the other measurements (Fig. 2, etc.) were of different stock, but litter mates. They were brothers of the seventh backcross generation of King inbred albinos with a dark-eyed stock, and were therefore practically homozygous.

III.

The behavior of a rat creeping upon an inclined plane shows one striking peculiarity. It is well known that during the upward locomotion of at least certain negatively geotropic animals the path of progression, especially at inclinations less than 90°, may not be exactly normal to the intersection of the creeping plane with the horizontal. As the inclination is made less, the deviation from the normal increases. This is very obvious in the rats. But there is to be added the further and very important fact that when a rat, at first placed head downward, or with body axis horizontal, orients upward it does so until a certain quite definite angle has been reached, *and then progresses in a straight line*. If creeping becomes interrupted, the rat may show "nervous" random movements of the head. In case these are directed downward, the rat continues creeping along the previous oriented path. But should they be directed upward, locomotion may be pursued at an angle which is exactly the converse of that at first followed. Thus if the angle of orientation was at first 72° to the left, brief creeping may be seen which is more or less irregular but which becomes definite again either at 72° ± to the left, or at 72° ± to the right (*cf.* Fig. 1). This clearly points to the limitation of geotropic orientation by a certain threshold determined through the distribution of the gravitational effect upon the two sides of the body. We shall have occasion to return to this point subsequently.

The results summarized in Table I are derived from twenty tests at each inclination, upon each of two rats from the same litter. Individual quantitative differences undoubtedly exist between diverse genetic strains, but since we are not concerned at the moment with this aspect of the matter we have restricted our account to illustrative material free from this source of confusion. The interpretation of data upon other individuals is entirely consistent with that here detailed. The entries in Table I concern (1) the angle of inclination (α) of the creeping plane to the horizontal; (2) the mean angle of orientation in the creeping plane (θ); and (3) the measure of the variability of θ, employing for this purpose the probable error ($0.8534\ \Sigma v/n\sqrt{n-1}$) expressed as a percentage of the mean.

It is apparent from Table I that the degree of upward orientation

(θ) increases steadily as the inclination of the creeping plane (α) is made greater; and also that the degree of scatter of the individual readings proportionately decreases—that is, the precision of the orientation is enhanced. The minimum inclination leading to a measurable effect lies between $\alpha = 10°$ and $\alpha = 15°$. At 15° the variability of the measurements of θ is disproportionately high, due presumably to the fact that the threshold effect is intrinsically variable from moment to moment. At values of $\alpha > 70°$, orientation is precisely upward ($\theta = 90°$).

TABLE I.

The mean angles of upward orientation (θ) of young rats during creeping upon a surface inclined at angles (α) with the horizontal, and the precision of the respective mean values of θ. The precision is expressed by the probable error as a percentage of the mean (which is equivalent to the coefficient of variation).

$\alpha°$	θ	Variability of θ
		per cent
15	32.6°	8.18
20	44.5°	2.27
25	52.9°	1.87
30	57.4°	1.70
35	64.0°	1.41
40	69.8°	1.18
50	77.9°	1.04
60	84.7°	0.529
70	88.3°	0.351

The extent of orientation (θ) is not directly proportional to the gravitional component in the creeping plane, but to its logarithm. The graph in Fig. 2 shows that the equation

$$\theta = K \log (\sin \alpha) \qquad (1)$$

gives a satisfactory account of the observations; the goodness of fit is probably due to the fact that the individuals used were very closely comparable.

The extent of orientation as a function of α has been measured in certain molluscs by Davenport and Perkins (1897–98) and by Kanda (1916). In the former paper figures are given for the amount of orientation (θ), corrected for random movement, which is visible in *Limax maximus* after 45 seconds exposure upon an

inclined plane. Essentially this method was also followed by Kanda (1916), who tabulated the percentage of *Littorina* individuals oriented upward after 1 minute exposure. At best, that is with full correction for movements not directed by geotropism, this procedure can give no quantitative expression for the geotropic excitation; for we should need to have, rather, measurements of the times required to produce a given amount of orientation, expressed either as a constant angle (θ) or as a certain percentage of individuals. For this reason little can be gotten from these data. But it is perhaps significant that the amount of orientation, when expressed in this way, increases more rapidly than log sin α (Fig. 3).

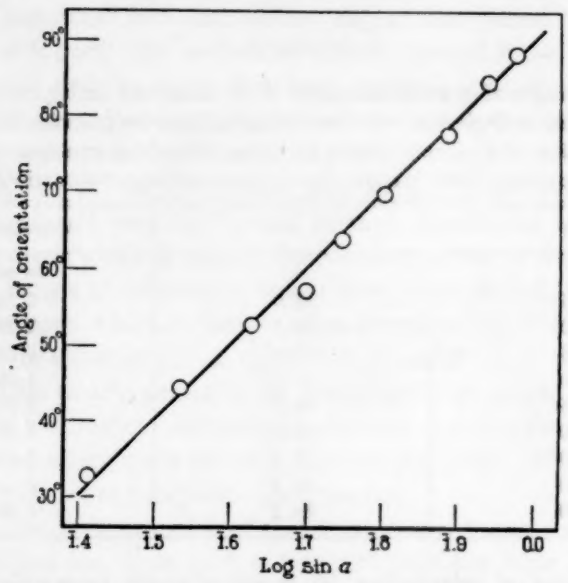

FIG. 2. The angle of upward orientation (θ) is directly proportional to log sin α, where α is the inclination of the creeping surface. With the exception of one point, the mean values of θ do not depart from the line drawn by more than their probable errors. (The points are averages of 40 measurements.)

This could be understood if the speed of orientation, so measured, should depend upon two things, namely speed of creeping and speed of turning, and if each of these separate elements of the act of orientation (Arey and Crozier, 1921; Crozier and Cole, 1923) should be proportional to log sin α. For the data of Davenport and Perkins this is very nearly true for values of α above 15°, and for Kanda's figures below $\alpha = 67° \pm$, but not very much weight can be given to the result.

The *speed* of upward creeping is frequently governed by the intensity of geotropic excitation, and in certain instances can be measured as an index of the effect

of gravity. Cole (1925–26) has done this with *Helix*, and concluded that the speed of movement, after orientation is attained, varies as sin α. For the rat, as we shall show presently, the velocity of upward movement decreases as log sin α increases. Question arises as to the existence of any real difference between the two cases. We believe that there is no real difference, because Cole's data show considerable deviation from (K) (sin α) at low values of α, and especially for the reason that the speed measured was that of the *vertical* ascension. The significance of the latter point lies in the fact that the extent of the average orientation (θ, in the

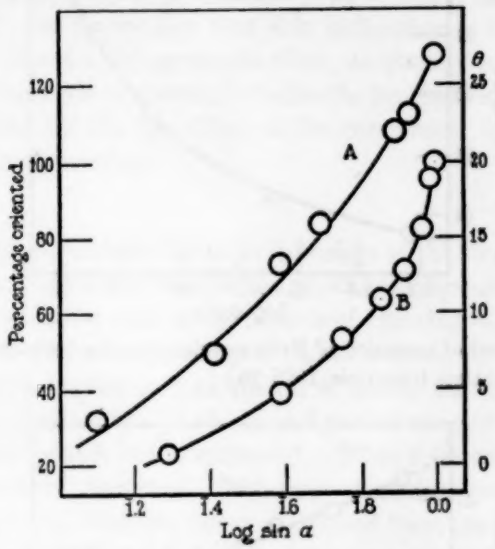

FIG. 3. Curve *A*. Angle of upward gravitational orientation (θ, corrected), for *Limax*, after 45 seconds; (data from Davenport and Perkins, 1897–98). Ordinate scale at the left.

Curve *B*. One series of measurements ("C") of percentages of oriented individuals after 1 minute (*Littorina*); ordinate scale at the right. (Data from Kanda, 1916.)

terms previously given), increases with α. Therefore the apparent speed of movement, measured as described, would probably be increased to an illegitimate extent. The data as given show that for *Helix* the "speed of vertical travel" increases faster than log sin α (Fig. 4).

We have somewhat regretted the form of the relation $\theta = K \log \sin \alpha$, although there are numerous instances of its applicability to other types of response (*cf.* Hecht, 1919–20; and many further cases), partly

because of its distressing generality, and partly because of its common association with the Weber-Fechner law. The latter interpretation is frequently misleading (*cf.*, especially, Hecht, 1923–24, 1924–25, and, for the case of phototropism under balanced illumination, Crozier,

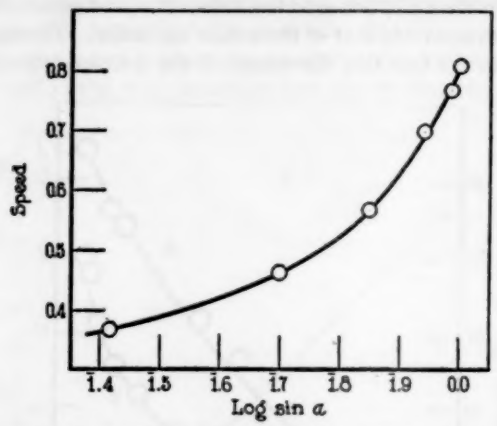

FIG. 4. The speed of ascension of *Helix* as related to the inclination (α) of the creeping surface. (Data from Cole, 1925–26.)

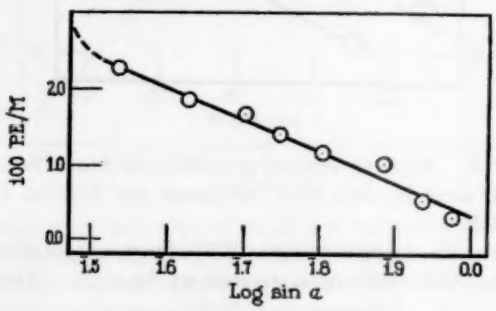

FIG. 5. The decrease in the coefficient of variability (P.E. as per cent of the mean), of the measured values of θ (= angle of upward orientation) is proportional to log sin α.

1926–27). But until more is known of this particular phenomenon we may accept the formula as a convenient empirical expression.

There is additional evidence of its applicability. If our conception of the orientation is correct, then as the gravitational effect is made greater the *precision* of the upwardly directed movement should be-

come correspondingly enhanced. This may be investigated by comparing the numerical expressions for variability of θ as measured at each value of α. If the reduction of variability (V) is proportional to the gravitational stimulus, then

$$-V = K \log \sin \alpha \qquad (2)$$

Fig. 5 shows that this relationship is well satisfied, with the exception of the relatively very large variability at $\alpha = 15°$; the deviation here is certainly due to the fact that this inclination is very close to the threshold value for any geotropic effect, as already stated. Thus not only the amplitude or extent, but also the precision of the orientation is determined by the logarithm of the component of gravity acting in the plane of creeping.

IV.

In searching for some clue as to the origin of the logarithmic relation between gravitational stimulus and geotropic response we have noted that if attention be paid to the process of creeping during orientation still another relationship emerges. Until a constant value of θ is attained upon a sloping surface the rat is chiefly *pulled* upward by the forward leg of one side of the body and *pushed* upward by the leg of the opposite side, which is less extended. When θ becomes constant the turning moment vanishes. We may consider, roughly and very crudely, that the orienting power is derived from the actions of levers on the opposite sides, and that the lever arm (x) on the "down" side is shorter than that (y) on the "up" side. Then the torque is responsible for turning upward. When the critical angle of orientation is exceeded, the locomotor action on the two sides of the body becomes equalized, so that if this value of θ is definitely exceeded (*i.e.*, beyond a fluctuating zone, of increasing smallness as α is made larger), the rat is no more constrained than upon a horizontal surface, and is free to turn, should it chance to do so, until an equivalent θ is reached on the other side of the perpendicular. We have already described precisely this behavior. In the line of progression defined by θ, the locomotor effectiveness of the opposed limbs is just barely identical. This means that if we assume the axes of the legs to have mean positions perpendicular to that of the body, then ($x \cos \theta - y \cos \theta$) exactly

balances the total downward pull of the animal's weight, where x and y are the "lengths" of the legs as levers on the two sides. Hence,

$$(x - y) \cos \theta = G \sin \alpha,$$

and

$$\frac{\cos \theta}{\sin \alpha} = \frac{G}{(x - y)}.$$

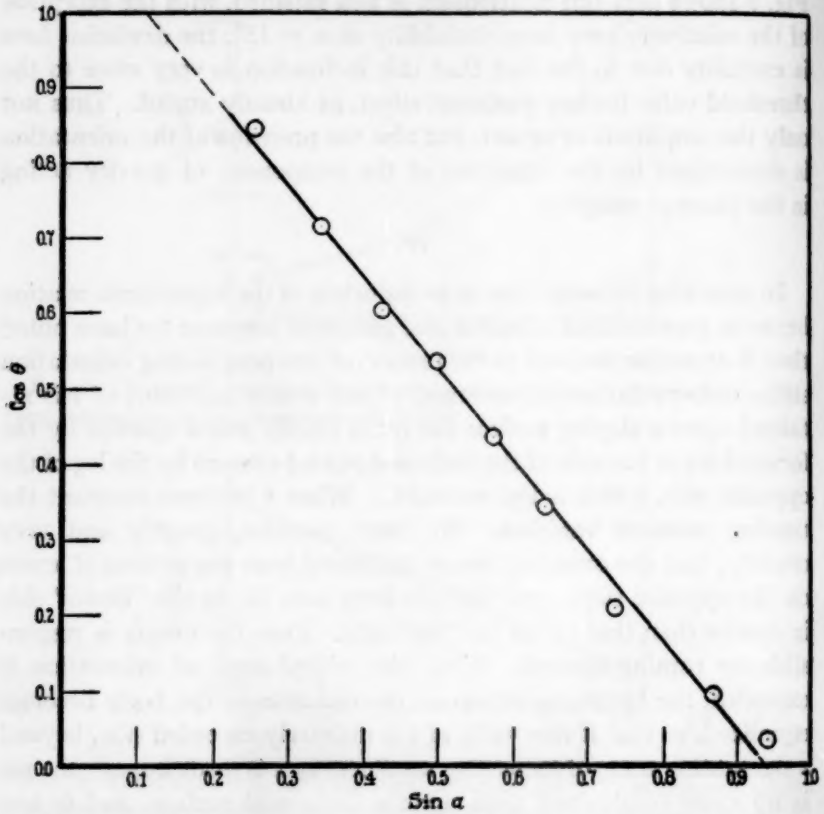

FIG. 6. The cosine of the angle of upward orientation (θ) decreases in direct proportion to the sine of the angle of inclination (α) of the creeping plane.

It was pointed out previously that the legs on the upward side are of course more extended; hence, $y > x$; and if $(x - y)$ is constant for all magnitudes of θ, we have

$$-\cos \theta = K \sin \alpha \qquad (3)$$

This relationship is in fact displayed, with unexpected exactness, as shown in Fig. 6, and gives an independent means of checking the significance of the data summarized in Table I. The constancy of the quantity labelled $(x - y)$, derived from the applicability of (3), at once suggests that the difference between the work done by the limbs of the two sides of the body in lifting the animal's weight must be reduced to constant fraction of the total before a stable orientation is attainable. This does not explain, of course, why the animal orients *upward* rather than downward, which may be determined by the inner ears; but it does explain why the amplitude of orientation attains its particular values as the inclination of the surface is varied, and in our opinion it gives an excellent illustration of the muscle-tension theory of orientation.

V.

From Fig. 6, by extrapolation to $\cos\theta = 1$, it is found that the ideal threshold value of α is at about 6.5°; at this point the component of gravity in the plane so slightly tilted is 0.113 G. Experimentally, so far as can be determined, the threshold angle is higher than this (10–15°). Hence we may assume, very roughly, because the extrapolation is probably invalid, that when the ratio of the loads on the two sides of the body falls below 10:9, no further orientation occurs. From the derivation of equation (3),

$$(x - y)\cos\theta = W\,G\sin\alpha,$$

$$\frac{\cos\theta}{\sin\alpha} = \frac{W\,G}{(x - y)},$$

it follows that if the weight, $W\,G$, be increased by attaching an additional load with thread to the animal's tail, then, at a given value of α and of θ, the product $(x - y)\cos\theta$ must be larger. The effect of adding such loads is to increase the magnitude of θ; hence it would be expected, from the formula, that $(x - y)$ must increase. The fact is that the locomotion is more labored with added weights attached, and the limbs, especially on the upward side, do become more extended—hence there is good evidence for the occurrence of a change corresponding to an increase in the value of $(x - y)$. The velocity of creeping is decreased in proportion to the added load.

It is of greater interest to see the effect of additional weights upon the extent of the upward orientation (θ). If the effect were a purely mechanical one we would expect to find θ increased in direct proportion to the added weight. But from equation (1),

$$\theta = K \log \sin \alpha,$$

we should expect the change to be such that

$$\theta = K (\log \sin \alpha + \log W \sin \alpha), \qquad (4)$$

where W is the added mass, and, when α is constant, θ should increase as log W. Fig. 7 shows that it does. The addition of as little as 1.0

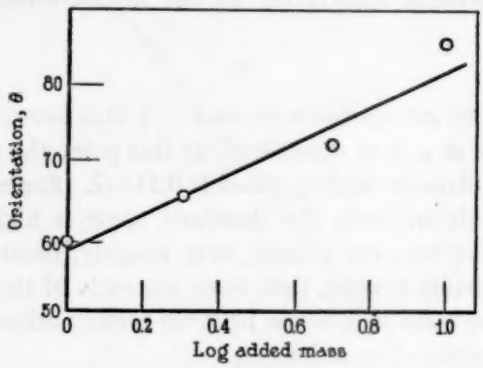

FIG. 7. The angle of upward orientation is proportional to the logarithm of the mass added to the tail of the rat, when the inclination of the creeping plane is constant.

gm. increases the upward orientation somewhat. It is worth while to investigate more fully the relations between θ, α, added load, and rate of locomotion, and in a later paper it is proposed to do so.

VI.

SUMMARY.

Young rats, old enough to creep well but before the eyelids are open, orient and move upward upon an inclined surface. The angle of geotropic orientation on such a surface (θ) is proportional to the logarithm of the component of gravity parallel to the inclined plane.

This result is compared with the scanty information available for other animals; there is indication that it may be generally valid. The precision of the orientation, measured by the percentage dispersion of the individual measurements, also increases in proportion to the logarithm of this component. The cosine of the angle of orientation decreases very nearly in proportion to the sine of the angle of inclination. A possible interpretation of this is given as involving the idea that upward orientation ceases when the differential pull of the body weight upon the opposed legs reaches a threshold value. Attaching weights (W) to the tail causes θ to increase, and in proportion to log W.

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THE PENETRATION OF BASIC DYE INTO NITELLA AND VALONIA IN THE PRESENCE OF CERTAIN ACIDS, BUFFER MIXTURES, AND SALTS.

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I.

INTRODUCTION.

It has been shown that the pH value of the cell sap plays[1] an important rôle in the accumulation of the basic dye, brilliant cresyl blue, in the living cell of *Nitella*, and in view of this it is important to study the changes in the rate of penetration produced by varying the pH value of the sap. Experiments of this sort, made by McCutcheon and Lucke,[2] and by the writer,[3] showed that the penetration of ammonia increases the pH value of the sap and decreases the rate of penetration of the dye.

The present paper deals with experiments on the rate of penetration of the dye in presence of acids and buffer mixtures. These experiments are of interest in connection with the hypothesis[1,4] that brilliant cresyl blue exists in aqueous solution in two forms, called for convenience DB and DS. DB, the form which predominates at higher pH values, represents a free base while DS exists predominantly at lower pH values and is a dissociated salt. A normal living cell of *Nitella* is assumed to be chiefly permeable to DB and only very slightly permeable to DS. The present problem is to find the nature of the factors controlling the penetration of DB.

[1] Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 561.
[2] McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.
[3] Irwin, M., *J. Gen. Physiol.*, 1925–26, viii, 147.
[4] Irwin, M., *J. Gen. Physiol.*, 1926–27, x, 75.

II.

Methods.

Only general methods will be given here: special methods for each set of experiments will be described in connection with the results.

The experiments were carried out in an incubator at 25°C. ± 0.5° having air holes through which diffused light entered.

Only living cells were used. In order to obtain cells in the same condition for experimentation uniformity as to length, thickness, and external appearance was attended to. In the case of *Nitella flexilis* the cells used were taken from the central portion of the plant, midway between the tip and the root. Seasonal changes bring about differences in the permeability of the cells, so that a series of comparative experiments were made on the same lot of cells collected within a short period (near New York in spring unless otherwise stated).

A control experiment was always carried out by removing cells directly from tap water and placing them in the same dye solution as in the case of the test experiment (in which the cells were given some special treatment before being placed in the dye). The rate of penetration obtained from the control experiment was used as a standard of comparison in order to determine the change in the rate of penetration of the dye caused by varying the media in which the cells were placed previous to exposure to the dye solution.

Every determination given represents an average of over 60 experiments and the probable error of the mean is in all cases less than 7 per cent of the mean.

In the case of *Valonia macrophysa* (collected in Bermuda), the procedure was as follows: The clusters of cells were pulled apart and the individual cells were allowed to stand in pans of sea water (which was changed daily) for over 2 weeks in the laboratory (exposed during the day to diffused light). During this period the cell wall at the point of detachment thickened somewhat. This precaution was taken to diminish irregularity in the rate of penetration and the susceptibility of cells to injury upon exposure to solutions.

Small cells (each having a volume of about 0.1 cc.) with one point of detachment, and having practically no attached cells, were chosen. Care was taken to remove adhering organisms or deposits from the surface of the cell.

The detection of an early stage of reversible injury is a very difficult matter, especially with *Valonia*. In the case of *Nitella* an increase in the rate of accumulation of the dye may serve as an indication of a preliminary stage of an injury under certain conditions but this does not seem to be markedly evident in *Valonia*. The exit of halides from the vacuole of *Nitella* or the entrance[5,6] of SO_4 into *Valonia* seems to indicate advanced stages of injury.

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[5] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, viii, 131.
Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925–26, ix, 255.

[6] Brooks, M. M., *Am. J. Physiol.*, 1926, lxxvi, 360.

Cells in good condition are turgid: as they become injured the turgidity diminishes. An experienced experimenter can, to a certain extent, predict the degree of resistance of the cells of *Nitella* and *Valonia* to experimental treatment by the turgidity as ascertained by touching them. This method was used by the writer as a rough guide to the condition of the cells but it does not serve to tell whether injury is reversible. The criterion of irreversibility of injury employed by the writer was as follows: cells which had been exposed to experimental conditions were replaced in the normal medium (*Nitella* in tap water, *Valonia* in sea water), and at intervals during 2 days the rate of mortality was compared with that of the control cells (without exposure to experimental conditions). The criterion of death for *Nitella* was a complete and permanent loss of turgidity, and for *Valonia* either a complete collapse of the cell or disarrangement of chlorophyll and its appearance in the vacuole, so that the greater part of the cell surface appeared colorless. Another test of the condition of the cells is to observe the length of time it takes for them to die in the experimental solutions.

It is not possible, however, to determine experimentally whether the cell was injured at the time of experiment, unless the injury happened to be irreversible. By using these tests an attempt was made to keep the cells uniform during the experiments.

The dye used was made by Grübler and was dissolved in buffer solutions (μ/150) in the case of *Nitella* and in sea water in the case of *Valonia*. The pH values of the solutions determined colorimetrically were checked as much as possible by means of the hydrogen electrode. Solutions were not stirred unless otherwise stated.

The determination of the concentration of the dye in the sap was made colorimetrically. With *Nitella*, the cell was gently wiped and was cut at one end, so that the sap could be squeezed out onto a glass slide. With *Valonia* the surface of the cell was punctured with a sharp capillary tube and the sap was drawn up from the vacuole into the tube, from which it was pushed out onto a glass slide. In both cases the sap was drawn up into capillary tubes and the color was matched with capillary tubes of the same diameter containing standard dye solutions.

To determine the pH value of the sap a definite volume was taken by filling a tube for 2 inches with the sap. Indicator solution was drawn up into another tube for a distance of $\frac{1}{20}$ of an inch. The contents of both these tubes were pushed out onto a glass slide and thoroughly mixed. This mixture was then drawn up into a capillary tube and the color matched with that of the capillary tube containing a mixture of standard buffer solution at a known pH value and the same amount of the indicator (the mixture was prepared in the same manner as in the case of the sap). Care was taken to have the least possible contamination of the sap by CO_2 from the breath of the experimenter, as well as to prevent escape of CO_2 into the air, as far as possible.

The color of the indicators changed on standing in a buffer solution containing artificial *Valonia* sap, and also on standing in the natural expressed sap, but the color of the indicators did not change during the time required to determine the pH value of the sap.

The salts in the sap of *Nitella* (about 0.1 M halides) do not seem to affect the indicator seriously, but those in the sap of *Valonia* (about 0.6 M halides) have a very definite effect. In view of the fact that we know so very little about the salt error in general, and possible specific effects of individual salts on these indicators, it will be necessary to study this question carefully before absolute pH values of the sap of *Valonia* can be given.

Another possible source of error in the case of *Valonia* is that the sap is so little buffered that an addition of indicator solution may bring about a change in the pH value of the sap; careful experiments must therefore be made to avoid this error. On the other hand, the sap of the *Nitella* used by the writer is buffered so that this source of error may be negligible. Since only approximate and relative values are desired the pH values of the sap of *Valonia* and *Nitella* given in this paper represent values without a correction for salt error, determined by means of one concentration of indicator dissolved in distilled water of pH 5.8 (approximately the pH value of the sap), or indicator dissolved in alcohol (methyl red). For one series of changes in the pH values only one indicator is used. For example, when experiments were made by exposing cells to a solution of NH_4Cl, brom-cresol purple was used: in the case of cells placed in acid solutions methyl red was used. Brom-cresol green was used to check the values obtained with methyl red, but in view of the fact that the color above pH 5.2 was not satisfactorily matched, only a very rough estimation of the pH value of the normal sap could be made by this indicator. Each indicator is taken from the same stock solution for each series of experiments.

III.

The Decrease in the Rate of Penetration of Dye When the pH Value of the Sap Is Lowered by Entrance of Acetic Acid.

The cells were divided into four lots. One lot was placed in an acetate buffer mixture at pH 5.1, and at the end of 10 minutes the pH value of the sap was compared colorimetrically with that of the normal cell sap. It was found to have decreased[7] from pH 5.5 (normal) to pH 4.9.

[7] It may be added here that these experiments show that acetic acid enters the vacuole rather easily from an acetate buffer mixture and decreases the pH value of the sap until the internal pH value is less than the external. This agrees with the results obtained by many investigators showing that weak acids enter the living cells. The writer's experiments also show that the pH value of the sap may be raised again when acetic acid is allowed to come out of the vacuole by placing the cells in a solution containing no acetic acid (the more alkaline the external pH value, the more rapid is the rate of exit of acetic acid from the vacuole).

The second lot of cells was placed in the acetate buffer mixture at pH 5.1, and after 10 minutes they were removed, wiped, rinsed for 5 seconds in phosphate buffer mixture at pH 6.6, wiped, and placed in fresh phosphate buffer mixture at pH 6.6. After 1 minute the cells were removed, and the pH of the sap was determined. It was found to be pH 5.2, which is 0.3 pH lower than that of the normal cell sap.

TABLE I.

Comparison of the amount of brilliant cresyl blue in the vacuole when the living cells of *Nitella* are placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after previous exposure to M/150 acetate buffer solution at pH 5.1 for different lengths of time. The rate of penetration of dye in the case of cells directly transferred from the tap water to the dye solution is used as the standard of comparison.

External solutions.		In tap water at pH 7.7.	In acetate buffer solution 5 sec.	In acetate buffer solution 1 min.	In acetate buffer solution 10 min.
When dye solution is not stirred or changed.	Amount of dye in sap.....	M 0.000073	M 0.000069	M 0.000069	M 0.000037
	Percentage decrease on basis of 0.000073 as standard.....		5 per cent	5 per cent	50 per cent
When dye solution is stirred and changed every 5 sec.	Amount of dye in sap.....	M 0.00012			M 0.000056
	Percentage decrease on basis of 0.00012 as standard....				47 per cent

The third lot of cells was first exposed to the acetate buffer solution at pH 5.1 for 10 minutes, after which they were removed, wiped, washed for 5 seconds in phosphate buffer mixture at pH 6.6, again wiped, and placed in the 0.00035 M dye solution at pH 6.6 (phosphate buffer mixture). After 1 minute they were removed from the dye solution and the concentration of the dye in the sap was determined colorimetrically, and was found to be 0.000037 M.

The fourth lot of cells was taken directly from the tap water (at pH 7.7) and placed in the same dye solution as the third lot of cells. At the end of 1 minute the concentration of the dye in the sap was found to be 0.000073 M.

Cells thus treated did not live so well as the control cells when replaced in tap water so that in all probability they were more or less injured, but during the experiment the actual appearance of the cells, in respect to chlorophyll arrangement and turgidity, seemed about the same as that of control cells except that the sap appeared slightly murky. Cells kept continuously in the acetate buffer solution began to die in about 3 hours, so that after an exposure of 10 minutes there may have been a very slight injury.

Thus these experiments show that the decrease in the pH value of the sap brought about by acetic acid may be associated with a decrease[8,9] in the rate of penetration of dye amounting to about 50 per cent, as shown in Table I.

This fact is of particular interest in connection with the theory[1,4] that the dye is chiefly in the form of free base (for convenience called DB), at high pH values, and that this alone can penetrate the proto-

[8] This decrease in the rate of penetration of dye is not due to the lowering of pH value of the external solution immediately surrounding the cell wall as a result of diffusion of acetic acid from the vacuole, because when the experiment is repeated by stirring the external solution, the relative amount of decrease in the rate is about the same as when the external solution is not stirred, as shown in Table I. Furthermore, this decrease is not caused by the adhering of acetic acid to the surface of cell wall in such a manner that it cannot be removed by washing and wiping before the cells are placed in the dye solution, because when the cells are placed in the dye solution, after they have been dipped in the acetate buffer solution only for 5 seconds or for 1 minute instead of 10 minutes, during which exposure the pH value of the sap remains normal, there is no decrease in the rate of penetration of dye, as shown in Table I.

[9] This result is contrary to the result obtained with Cambridge *Nitella* previously described (Irwin, M., *J. Gen. Physiol.*, 1925–26, ix, 566, Foot-note 11) where an increase in the rate of penetration took place, but the extent of this increase was not so great (about 25 per cent). Since this work on Cambridge *Nitella* was done in midwinter, the experiments were repeated with the cells obtained in the summer, and it was found that in the majority of cases a decrease took place (about 25 per cent), which is less than the decrease in the case of New York *Nitella*. Such a difference in the behavior of cells may be due to the difference in the condition of the protoplasm.

plasm and enter the vacuole, and that the extent of accumulation of the total dye is dependent on the extent of change of this form, DB, on entering the vacuole into another form, DS, which cannot pass through the protoplasm. In that case we might expect the rate of penetration into the vacuole to be increased when the pH value of the sap is decreased, since with this decrease in the pH value of the sap the ratio of DB/DS decreases in the sap so that as DB enters the vacuole more of it will change to DS, thus causing more DB to enter. But since the experimental results give evidence to the contrary it is evident that the factor which controls the rate of penetration of dye into the vacuole cannot be wholly dependent on the condition of the sap. Under the present experimental conditions the rate of penetration of the dye must be controlled primarily by the effect of the acetate buffer on some other part of the cell. A series of experiments was therefore undertaken to determine the cause of this decrease in the rate of penetration of dye into the vacuole.

IV.

Can the Decrease in the Rate of Penetration of the Dye be Produced without Change in the pH Value of the Sap?

If the theory[4] outlined in Sections I and III were correct we might assume that the decrease in the rate of penetration of dye associated with a decrease in the pH value is due to a change either at the surface or inside the protoplasm caused by the acetate buffer mixture. In that case we might very well expect a decrease in the rate of penetration when the cells are exposed[10] to the solution only long enough for the protoplasmic surface or the interior of the protoplasm to be affected before a change in the pH value of the sap occurs. Unfortunately it is not possible to use the acetate buffer solution for this purpose since the pH value of the sap changes after a very few

[10] The detailed description of the method of experimentation will be omitted hereafter since it is given in Section III. It may be repeated here that in all cases the cells were washed for about 5 seconds in a buffer solution at the same pH value as that of the dye solution before they were placed in the dye solution and the cells were invariably wiped before they were placed in any solution. Cells were exposed for 1 minute in the solution of dye, 0.00035 M made up with phosphate buffer mixture at pH 6.6 unless otherwise stated.

minutes exposure of the cells to the solution, even at the highest possible pH value (pH 5.4, M/150 acetate buffer mixture). For this reason it was necessary to expose the cells to a phosphate buffer solution at pH 5.4 for 10 minutes, in which the pH value of the sap remained unchanged, and to compare the rate of penetration of dye in the case of cells thus treated with the rate in the case of cells previously exposed for the same length of time in an acetate buffer solution at pH 5.4 where the pH value of the sap decreased from 5.5 (normal) to 5.0. As shown in Table II, there is about the same

TABLE II.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when the cells are placed in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) after a previous exposure of the cells for 10 minutes either to the M/150 acetate buffer solution (when the pH of the sap is decreased) or to the M/150 phosphate buffer solution (when the pH value of the sap is not decreased).

External solutions.	In tap water at pH 7.7.	In acetate buffer solution at pH 5.4.	In phosphate buffer solution at pH 5.4.
	M	M	M
Amount of dye in sap	0.000073	0.000039	0.000041
Percentage decrease on basis of 0.000073 as standard		47 per cent	44 per cent

amount of decrease[11] in the rate of penetration whether the pH value of the sap is lowered or remains normal. The mortality of the cells thus treated is lower than that of the cells exposed to the acetate buffer mixture.

[11] Since there is about the same amount of decrease in the rate of penetration of dye whether the pH value of the sap is decreased or not, such a decrease cannot be due primarily to the decreasing of the pH value of the film[4] of liquid between the protoplasmic surface and the cell wall as result of diffusion of acetic acid from the vacuole into the film. This film is the only part of the external system which affects penetration since it alone determines the number of dye molecules striking the protoplasmic surface. The condition of the external solution may be regarded as of importance only in so far as it affects this film.

V.

Is the Decrease in the Rate of Penetration of the Dye Due to the Effect of H Ions on the Surface or to Their Penetration (as Ions) or to the Entrance of Acids in Undissociated Form?

The decrease in the rate just described was about the same whether the pH value of the sap was lowered or not, and this suggests that the decrease in the rate might be due to the direct action of H ions on the surface or their penetration as ions when the pH value of the external solution changed from pH 7.7 (tap water) to pH 5.4 (buffer solutions). If this assumption were correct we might expect the rate of penetration to be about the same whether the cells were previously exposed to tap water, to phosphate, or to borate buffer solutions at pH 7.7 providing equal numbers of hydrogen or hydroxyl ions enter in each case.

In order to test this the rates of penetration of dye were compared among the three groups of cells previously placed[10] for 10 minutes (1) in tap water (control), (2) in phosphate buffer solution, and (3) in borate buffer solution, all at pH 7.7, and it was found (as shown in Table III) that with phosphate buffer solution there was about 30 per cent less dye in the vacuole than in the case of the control, and with borate buffer about 13 per cent less dye (which may not be significant since the probable error of the mean is rather high).

The experiments were extended to higher pH values, pH 8.1 and 7.3, and it was found, as shown in Table III, that the rate of penetration of dye is again lower in the case of cells previously exposed[10] to the phosphate buffer solution than that in the case of cells exposed to the borate solution. Such a difference in behavior between the borate and the phosphate buffer mixtures cannot be due to the effect of H or OH ions as such on the cell, since the pH value is the same in both these solutions.

Other experiments are therefore needed to determine just what causes this difference.

This difference between the phosphates and the borates, as affecting the rate of penetration of dye, is not due to the difference in the effect[12]

[12] It is not possible, unfortunately, to determine if there is an effect of acetate buffer mixture on the dye, since it is impossible to determine the penetration of dye at a pH value lower than pH 6.2 in the case of *Nitella*.

of these buffer mixtures directly on the dye, as is proved by the fact that when the cells are transferred[10] directly from the tap water to

TABLE III.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella*, when cells were previously exposed to M/150 borate and phosphate buffer solutions at different pH values for 10 minutes after which they were placed for 1 minute in 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture).

External solutions.	In tap water at pH 7.7.	In phosphate buffer solution at pH 7.7.	In borate buffer solution at pH 7.7.	In phosphate buffer solution at pH 8.1.	In borate buffer solution at pH 8.1.
	M	M	M	M	M
Amount of dye in sap	0.000079	0.000055	0.000069	0.000059	0.000079
Percentage decrease on basis of 0.000079 as standard		30 per cent	13 per cent	26 per cent	0 per cent

External solutions.	In phosphate buffer solution at pH 7.3.	In borate buffer solution at pH 7.3.	In phosphate buffer solution at pH 6.6.	In borate buffer solution at pH 8.7.	
	M	M	M	M	
Amount of dye in sap	0.000052	0.000069	0.000048	0.000079	
Percentage decrease on basis of 0.000079 as standard	35 per cent	13 per cent	39 per cent	0 per cent	

TABLE IV.

Comparison of the amount of brilliant cresyl blue in the sap after 1 minute in 0.00017 M dye solutions at pH 7.7 made up with different buffer mixtures (M/150).

External dye solutions.	Borate buffer mixture.	Phosphate (ordinary) $Na_2HPO_4 + KH_2PO_4$.	Phosphate (lacking K) $Na_2HPO_4 + NaH_2PO_4$.
	M	M	M
Amount of dye in sap	0.00041	0.00035	0.00040

the dye solution at pH 7.7 made up (1) with borate buffer mixture and (2) with phosphate buffer mixture, the rate of penetration of the dye is the same, as shown in Table IV.

This difference furthermore is not due to a specific action of the K in the phosphate buffer mixture since the experiments were repeated[10] with the solution made up with Na_2HPO_4 containing NaH_2PO_4 instead of KH_2PO_4, at pH 5.4 and 7.7 and the same result was obtained, as shown in Table V.

TABLE V.

Comparison of the amount of brilliant cresyl blue in the vacuole of living cells of *Nitella* after 1 minute exposure to 0.00035 M dye solution at pH 6.6 (M/150 phosphate buffer mixture) following a 10 minute exposure to the M/150 phosphate buffer mixtures consisting of Na_2HPO_4 and either KH_2PO_4 or NaH_2PO_4.

External solutions.	In tap water at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 7.7.	In Na_2HPO_4 + NaH_2PO_4 at pH 7.7.	In Na_2HPO_4 + KH_2PO_4 at pH 5.4.	In Na_2HPO_4 + NaH_2PO_4 at pH 5.4.
	M	M	M	M	M
Amount of dye in sap	0.000084	0.000055	0.000059	0.000048	0.000048
Percentage decrease with 0.000084 as standard		33 per cent	29 per cent	42 per cent	42 per cent

TABLE VI.

Comparison of amount of brilliant cresyl blue in the vacuole when cells of *Nitella* (autumn) are placed in 0.00004 M dye solution (stirred) at pH 7.7 (M/150 borate buffer) for ½ minute, after they have been exposed for 10 minutes to various solutions.

External solutions.	Tap water pH 7.7.	Boric acid pH 4.8.	Hydrochloric acid pH 4.8.	Phosphoric acid pH 4.8.	Phosphate buffer pH 5.4.
	M	M	M	M	M
Dye in sap	0.000072	0.000076	0.000069	0.000058	0.000042
Percentage decrease on basis of 0.000072 as standard		5 per cent increase (?)	5 per cent	20 per cent	42 per cent

The inhibiting effect of phosphate buffer mixtures is greater the lower the pH value, as shown in Table III. It may be that this is due to the greater amount of phosphoric acid present in the buffer mixture, if we assume that as a weak acid it penetrates the protoplasm as undissociated molecules and dissociates after entering and

lowers the pH value of the protoplasm, so that when cells are subsequently placed in a dye solution there will be less DB (since DB changes to DS more at a low pH value) in the protoplasm than in the case of the control cells which are transferred directly from tap water to the dye solution. The rate of penetration of DB from the protoplasm to the vacuole will therefore be less than in the case of the control cells.

This assumption[13] is partly supported by the following result. When the cells[13] are exposed for 10 minutes to three separate solutions, (1) hydrochloric acid, (2) boric acid, (3) phosphoric acid, all at pH 4.8, and then placed in the dye solution[13] (borate) for ½ minute, the rate of penetration of dye (as compared with that of the control cells, which are transferred directly from tap water to the same dye solution) in the case of hydrochloric acid and boric acid is about the same as that of the control. This indicates that H ions do not affect[13] the cell and that if boric acid enters the cell as undissociated molecules it does not afterward dissociate sufficiently to lower the pH value to any appreciable degree. Phosphoric acid behaves differently in that the rate of penetration of the dye in the case of the cells exposed previously to this acid is found to be about 20 per cent lower than that of the control, which indicates that phosphoric acid enters the

[13] There are several other possible explanations, for example:

(1) On the basis that phosphoric acid enters more rapidly than boric. We are unable to prove this experimentally, for which reason the explanation described in the text is used instead.

(2) On the basis that a weak acid enters the protoplasm as undissociated molecule and by dissociating lowers the pH value of the protoplasm and that when such cells are removed from the buffer solution to the dye solution, the weak acid diffuses out from the protoplasm into the film of liquid between the protoplasmic surface and the cell wall, and lowers the pH value of the film thereby decreasing the ratio of DB/DS in the film. This will explain the difference between boric acid and phosphoric acid, in that boric acid does not change the pH value of the film since it is too weak an acid, while phosphoric acid is sufficiently strong to bring about this change. But this assumption is not so satisfactory as the one described in the text when we consider the fact that there is an inhibiting effect on the rate of penetration of dye even with cells previously exposed to a phosphate buffer solution at pH 8.1 and then placed in dye solution at much lower pH value (pH 6.6). In such a dye solution one would expect further entrance of phosphoric acid into the cell, rather than exit of the acid from the protoplasm to the exterior of the cell. These cells were collected in autumn.

The dye solution was stirred. At a lower pH value both phosphoric acid and hydrochloric acid have an inhibiting effect which is greater in the case of the former.

protoplasm and then dissociates sufficiently to lower the pH value or else that it has a specific effect on the surface (Table VI).

The cause of the decrease brought about by the phosphate buffer mixture may be threefold, (1) due to undissociated phosphoric acid, (2) due to the Na and K salts present in the buffer mixture, and (3) due possibly to certain anions.

It may be of interest to add here the following. When cells (collected in Cambridge) are exposed for 10 minutes to solutions at different concentrations (0.05 M to 0.006 M) of NaCl, LiCl, KCl, Na_2SO_4, and $NaNO_3$ made up in distilled water, after which they are washed in distilled water for 5 seconds, wiped, and are placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) for 1 minute, the rate of penetration is considerably decreased as compared with the control. If cells are placed directly for 1 minute (without such treatment) in 0.00014 M dye solution at pH 7.7 (borate buffer mixture) containing any one of these salts, the rate is found to be slightly higher than in the case of cells placed in dye solution containing no salt.

Solutions of $MgCl_2$, $MgSO_4$, $CaCl_2$, $LaCl_3$, and $LaNO_3$ all behave alike, in that when cells are exposed to these solutions for 10 minutes and then transferred to the dye solution, the rate of penetration of dye is about the same as the control. When cells are placed without such treatment in dye solutions containing any one of these salts ($LaCl_3$ omitted), the rate is found to be somewhat higher than that of the control.

Thus there is evidence for the inhibiting effect[14] of the salts with

[14] The experiments described in the text (see Table III) show that the borate buffer mixtures have no inhibiting effect on the rate of penetration of dye. In view of the fact that the borate buffer mixtures at higher pH values contain a considerable amount of Na, there is an apparent discrepancy between the results obtained in this case and those in the case of NaCl solutions in which there is a considerable inhibiting effect due to the presence of Na (this discrepancy was mentioned in the writer's previous paper (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, No. 1)). This, however, may be due to the fact that in the case of cells previously exposed to the borate buffer mixtures the dye was made up with phosphate buffer mixtures which seem to diminish the inhibiting effect of Na, while in the case of cells previously exposed to NaCl solutions the dye used was made up with borate buffer mixture which does not seem to have this effect. The following experiments may make this clear. When cells previously exposed to (1) 0.01 M NaCl and (2) to 0.005 M sodium borate solutions for 10 minutes were washed for 5 seconds in distilled water, wiped, and placed in 0.00014 M dye solution at pH 7.7 (borate buffer mixture), there is a considerable inhibiting effect

monovalent base cations which is not easily reversible, since the effect may be brought about by placing the cells in 0.01 M NaCl solution for 5 minutes, but this effect does not disappear after the cells have been transferred to distilled water and left for over an hour. This effect, however, may be readily removed if cells are placed in a solution of salt with bivalent or trivalent cations, such as $MgCl_2$ and $LaCl_3$ at certain concentrations.

Further experiments are being carried out on this subject by the writer.

VI.

Experiments on Valonia.

The experiments have been repeated with *Valonia macrophysa* but the results described below are approximate and show only relative values, owing to the fact that the pH value of the sap cannot be accurately determined without special experiments and that the sea water not only shifts the dissociation constant considerably, but seems to change the nature of the dye, especially at lower pH values.

A. The Effect of Aqueous Ammonia (Free and Combined).—Since the method of determining the change in the pH value after placing cells in solutions has been described in detail in Section II, it will be omitted here. When cells of *Valonia* were placed for 1 hour in sea water containing 0.003 M NH_4Cl solution, the pH of the sap increased from 6 (normal) to 6.6 (determined colorimetrically by using bromcresol purple). When such cells were replaced in sea water and left for 1 hour the pH value decreased from 6.6 to 6.1.

One group of cells was placed in sea water containing 0.00035 M dye, a second group in sea water containing 0.003 M NH_4Cl and

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which is slightly greater with (1) than with (2). If such cells (1) and (2) are placed in 0.00014 M dye solution at pH 7.7 (phosphate buffer solution) they show no inhibiting effect at all.

At a higher concentration of NaCl (0.05 M) this inhibiting effect is not removed in 0.00014 M dye made up with phosphate buffer mixture at pH 7.7.

The inhibiting effect of previous treatment with the phosphate buffer mixture at pH 5.4 (Table II) is increased in 0.00014 M dye solution at pH 7.7 made up with borate buffer mixture.

0.00035 M dye. A third group of cells was first exposed for 1 hour to sea water containing 0.003 M NH_4Cl and then transferred to the dye solution used in the case of Group 1. After 1 hour there was a decided decrease in the rate of penetration of dye in the case of cells placed in the dye solution containing NH_4Cl (Group 2) and also in the case of cells previously exposed to NH_4Cl solution (Group 3), as compared with the control (Group 1). These results show that the presence of ammonia in the cell brings about a decrease in the rate of penetration of dye. Whether this decrease is entirely due to the increase in the pH value of the sap in the presence of ammonia or due partly to the former and partly to the presence of ammonia in the protoplasm (at the surface or the interior), it is not possible to determine. These results confirm those obtained with *Nitella*[3] (see Section I).

B. Effect of Acetic Acid and HCl at pH 5.9.—Let us first see if the same results may be obtained as with *Nitella* when the pH value of the sap is decreased by entrance of acetic acid. When cells were placed in sea water containing acetic acid at pH 5.9, the pH value of the sap decreased in 1 hour from 5.5 (normal) to 4.8 (methyl[15] red used as an indicator). The pH value of the sap thus decreased was found to be raised to the normal when such cells were placed in sea water for 20 minutes. When cells whose pH value had been thus decreased were placed for 20 minutes in sea water containing 0.00035 M dye, the amount of dye in the sap was less[16] than in the sap of cells transferred directly from the sea water to the same dye solution (control). These experiments show that there is a decrease in the rate of penetration of dye when the pH value of the sap is decreased

[15] Difference between the determination of the pH value of the sap made with brom-cresol purple and with methyl red lies in the fact that the effect of salt on the indicator is not corrected. The explanation of the use of the indicators is described in Section I.

[16] Brooks exposed cells of *Valonia macrophysa* to sea water (1) containing NH_4Cl until the pH value of the sap increased, and (2) containing CO_2 until the pH value of the sap decreased, after which they were placed in sea water containing 2, 6, dibromophenol indophenol, and found that the rate of penetration of dye decreased with (1) and increased with (2). She interprets these results on the basis that the rate of penetration of dye is affected by the change in the pH value of the external solution surrounding the cell as a result of diffusion of (1) NH_4Cl and (2) CO_2 from the vacuoles. (See Foot-note 6.)

by entrance of acetic acid, which agrees with the results discussed in Section III on *Nitella*.

Let us now see if a decrease can be brought about without a change in the pH value of the sap. Cells were exposed for 1 hour to sea water containing HCl at pH 5.9, after which they were placed in 0.00035 M dye for 20 minutes. The rate of penetration in this case was found to be less than the control but the extent of decrease in the rate is not so great as it was in the case of cells exposed to acetic acid.

When cells are placed for 1 hour in sea water containing 0.0007 M dye at pH 5.9, (1) containing acetic acid and (2) containing HCl, the rate of penetration was found to be higher with acetic acid than with HCl.

C. Effect of Sea Water at pH 6.5 Containing either Acetic Acid or HCl (No Change in the pH Value of the Sap).—The question now arises as to what will happen if we put cells in sea water containing acetic acid at a pH value at which there is no decrease in the pH value of the sap. Cells were placed in sea water at pH 6.5 containing acetic acid for 1 hour after which they were transferred to sea water containing 0.00035 M dye for 20 minutes: the pH value of the sap remained normal. When the rate of penetration of dye in the case of the cells thus treated was compared with that of the control (cells directly removed from the sea water and placed in the same dye solution), it was found to be the same. In the case of the cells previously exposed to sea water containing HCl at pH 6.5 the rate of penetration of dye was also found to be the same.

Cells placed in 0.00017 M dye in sea water at pH 6.5 containing (1) acetic acid and (2) HCl, showed no difference in the rates.

Thus these experiments show that in the case of *Valonia* also the rate of penetration of dye may be retarded when (1) the pH value of the sap is decreased in presence of acetic acid, and (2) the pH value of the sap is increased in presence of NH_3, when cells are exposed to these solutions before they are placed in the dye solutions.

SUMMARY.

When living cells of *Nitella* are exposed to an acetate buffer solution until the pH value of the sap is decreased and subsequently placed in a solution of brilliant cresyl blue, the rate of penetration of dye into the vacuole is found to decrease in the majority of cases,

and increase in other cases, as compared with the control cells which are transferred to the dye solution directly from tap water. This decrease in the rate is not due to the lowering of the pH value of the solution just outside the cell wall, as a result of diffusion of acetic acid from the cell when cells are removed from the buffer solution and placed in the dye solution, because the relative amount of decrease (as compared with the control) is the same whether the external solution is stirred or not.

Such a decrease in the rate may be brought about without a change in the pH value of the sap if the cells are placed in the dye solution after exposure to a phosphate buffer solution in which the pH value of the sap remains normal. The rate of penetration of dye is then found to decrease. The extent of this decrease is the greater the lower the pH value of the solution.

It is found that hydrochloric acid and boric acid have no effect while phosphoric acid has an inhibiting effect at pH 4.8 on stirring.

Experiments with neutral salt solutions indicate that a direct effect on the cell (decreasing penetration) is due to monovalent base cations, while there is no such effect directly on the dye.

It is assumed that the effect of the phosphate and acetate buffer solutions on the cell, decreasing the rate of penetration, is due (1) to the penetration of these acids into the protoplasm as undissociated molecules, which dissociate upon entrance and lower the pH value of the protoplasm or to their action on the surface of the protoplasm, (2) to the effect of base cations on the protoplasm (either at the surface or in the interior), and (3) possibly to the effect of certain anions. In this case the action of the buffer solution is not due to its hydrogen ions.

In the case of living cells of *Valonia* under the same experimental conditions as *Nitella* it is found that the rate of penetration of dye decreases when the pH value of the sap increases in presence of NH_3, and also when the pH value of the sap is decreased in the presence of acetic acid. Such a decrease may be brought about even when the cells are previously exposed to sea water containing HCl, in which the pH value of the sap remains normal.

The writer wishes to thank Miss Helen McNamara for her faithful assistance in carrying out the experiments.

THE RÔLE OF CERTAIN METALLIC IONS AS OXIDATION CATALYSTS.

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It has long been known that metals in some way are very closely connected with the processes of respiration and oxidation in both plants and animals, and the mode of their action has been the subject of a great deal of research and discussion. The most important of these metals is undoubtedly iron, and the recent work of Warburg (1925) and Meyerhof (1924) has gone far toward elucidating its mode of action.[1] Iron as an oxygen carrier is present in the hemoglobin of the blood of mammals and has been the subject of much investigation. It is not within the province of this paper, however, to discuss this aspect of the matter, treatment of which may be found in text-books of general physiology.

Next to iron, in occurrence and in importance, is copper, although the latter has not received the careful and attentive research which has been bestowed on the former.[2]

[1] The book by Meyerhof (Chemical dynamics of life phenomena, 1924) contains an excellent summary of investigations up to a very recent date. Reference may also be made to the individual papers of these authors, and particularly, in English, of Warburg (1925). Crozier (1924–25) has analyzed the data of various investigators who have worked with oxidation systems, from the point of view of the effect of temperature. He finds that many oxidation reactions, including several which are undoubtedly catalyzed by iron, have a critical thermal increment close to 16,000. Hecht (1925–26) explains on this basis the latent period of the photic response in *Ciona*, the critical thermal increment of which is nearly 16,000, as a reaction catalyzed by iron and probably an oxidation. The fact that many oxidation systems and many reactions catalyzed by iron have the same increment may be taken as further evidence that iron is intimately connected with oxidation catalysis.

[2] The toxic effects of copper have been widely observed and discussed (see papers by Cook, 1925–26). In fact, the striking and obvious toxic action of copper has more or less obscured the possibility that this element may be of fundamental importance in the catalysis of normal oxidation reactions.

That copper occurs normally in a large number of organisms has been shown by Maquenne and Demoussy (1920) for plants and Muttkowski (1920–21) and Rose and Bodansky (1920) for animals. In animals it is usually found in the hemocyanin, or oxygen-carrying fluid, of arthropods and crustaceans. Hemocyanin is considered analogous to hemoglobin, with copper taking the place of the iron. Henze (1904–05), working on octopus blood, decided that oxidations were catalyzed by the copper. Alsberg and Clark (1914), using *Limulus*, concluded that "With the aid of copper, oxygen may, perhaps, be transferred catalytically within the organism." Glaser (1923) found considerable copper in *Arbacia* eggs; he thinks its function is partially to inactivate certain enzymes.

Of the other metallic elements manganese (Bertrand, 1897, and later papers) is the only one which has been mentioned as a substitute for iron and copper. McHargue (1926) states that manganese is found very frequently in chlorophyl-bearing tissues and assigns to this metal an important rôle in photosynthesis. In molluscan bloods Mn may appear to take the place of Cu. However, its function, if not its occurrence, is rather problematical, and it does not take rank in prominence with the other two. Some reasons will be advanced, based on the present work, for believing that iron and copper, if not the only two elements possible, are nevertheless the two elements which are, chemically, especially well adapted to the rôle of oxidation catalysts.

One method of approach to the problem of biological oxidations is the measurement of respiration in organisms. Another is the attempt to duplicate, as far as possible, the conditions existing in the cell by means of inorganic chemical systems where the conditions may be controlled. The first method has led to the conclusion that iron, and to a lesser extent copper, is the catalytically active substance. The second method is more apt to furnish data regarding the mechanism of the reactions involved and is the one used in the present investigation.

II.

In order to duplicate the essential conditions in living systems it is necessary to have an oxygen-rich substance (a peroxide), a catalyst, and an easily oxidizable substance. Ray (1923–24) has proceeded according to this principle and has investigated the system iron-hydrogen peroxide-unsaturated fatty acids from the point of view of

the effect of anesthetics. It was thought best here to use a combination which reacted quite rapidly, and therefore pyrogallol was used as the oxidizable substance. One of the end-products of the reaction is carbon dioxide, the rate of production and amount of which may be accurately measured.

The experimental measurements were made with an Osterhout respiration machine the principles of which have been described in an earlier paper (Cook, 1925–26). Briefly, there is a closed system of tubes through which air is forced by a pump, from a reaction chamber to a tube containing an indicator (phenolsulfonphthalein) and thence by another route to the reaction chamber again. The indicator is decolorized by the carbon dioxide and the color is restored by switching the current of air through a U-tube containing sodium hydroxide.

In carrying out the present series of experiments a definite amount of pyrogallol was dissolved in water in a large test-tube (the reaction chamber); the tube was placed in its proper position in the circuit of the machine, and the carbon dioxide present in the solution was cleared out. Then through a separatory funnel a mixture of the metal salt and hydrogen peroxide was run into the reaction chamber. These two constituents were mixed *immediately* before being run in, so that there might be as little reaction between them as possible before striking the pyrogallol solution; the error here involved is entirely negligible because the reaction between the metal and the peroxide is relatively slow. Then the machine was started and the carbon dioxide produced was measured practically from the start of the reaction.

In order that there might be no possibility of the walls of the container exercising a catalytic effect, or otherwise disturbing the reaction, the inside of the reaction chamber was coated with paraffin and the coating renewed frequently. Furthermore, the indicator solution was replaced after every experiment in order to guard against any contamination by volatile organic acids which might be produced during the oxidation of the pyrogallol. Finally the reaction chamber was placed in a water bath and the temperature was kept uniformly at 25°C. in all the experiments here reported.

Unless the concentration of the reactants was purposely varied the mixtures were made up by dissolving 0.1 gm. of pyrogallol in 44 cc. of water (distilled) to which was added 1 cc. of hydrogen peroxide and 10 cc. of the metal salt in the desired concentration. The hydrogen peroxide was all taken from the same bottle and retained its strength at approximate constancy throughout the entire series of experiments.

Since the hydrogen ion concentration of the medium affects the rate of oxidation of pyrogallol it was ascertained colorimetrically that a mixture of 0.1 gm. of pyrogallol and 1 cc. of the peroxide in 44 cc. of water has a pH of approximately 5. Since most of the metal salts here used have an acid reaction there is no doubt that all the present experiments were performed in an acid medium. The pyrogallol

will not absorb oxygen as rapidly in an acid as in an alkaline medium, but since the hydrogen ion concentration did not vary to any great extent, and always remained on the acid side of neutrality, the relative values obtained are not invalidated. It would of course be impossible to conduct experiments by the indicator method with an alkaline medium in the reaction chamber.

It is customary when using the indicator method with an organism to express the results as a rate curve based on the normal rate of respiration as 100 per cent. Such a procedure is impossible here since there is no "normal rate." In fact there is no production of carbon dioxide previous to the start of the reaction.[3] It is necessary, therefore, to find some other method of expressing the results. This may be done by using the absolute amounts of carbon dioxide produced. Ray (1923–24) has developed this method with the assistance of E. J. Cohn and has calculated the actual quantity of the gas. Here it is not essential to know the actual quantities in mg. Arbitrary units are satisfactory since all the results are relative and may be compared with each other even if we do not know the exact amounts. If the same amount of sodium bicarbonate is always present in the indicator solution and the buffer standards always remain constant, then it will always take the same amount of carbon dioxide to decolorize the indicator. We may then take this amount as a unit and calculate the number of units produced in a given time, or the length of time to produce 1 unit. But we must remember that the reaction is also proceeding during the time that the color of the indicator is being restored and include this time. For example, if it takes 30 seconds to decolorize and 30 seconds to restore the color then 2 units of carbon dioxide are formed per minute. Or if in 10 minutes there are four periods of decolorization 2 minutes long and four periods of 30 seconds to restore the color, then 5 units will have been formed during the 10 minutes.

Using this method the total amount of carbon dioxide produced can be plotted against the time and an integral curve obtained. If the equation of this curve is known then the rate curve can be derived therefrom.

III.

Since this investigation has been primarily on the effect of copper, this element will be treated in a separate section. Fig. 1 shows typical curves obtained with 1 cc. of hydrogen peroxide, 0.1 gm. of pyrogallol, and various concentrations of copper chloride, in 44 cc. of water. The curves represent the total amounts of carbon dioxide produced, plotted against time. The slope of such a curve at any

[3] Pyrogallol is oxidized by hydrogen peroxide and metal salts separately at such a slow rate (if at all) that it cannot be detected by this method. Hence we may say for practical purposes that there is no production of carbon dioxide unless all three constituents are present.

point indicates the rate of production of carbon dioxide. It will be seen that with very dilute copper the rate is at first relatively rapid, but soon falls off until it approaches zero. With concentrated solutions the rate decreases continually, but since the earlier portion of the curves is most important it was not considered necessary to follow the course of the reaction entirely to completion. In fact to do so with concentrated copper solutions would require many hours if not

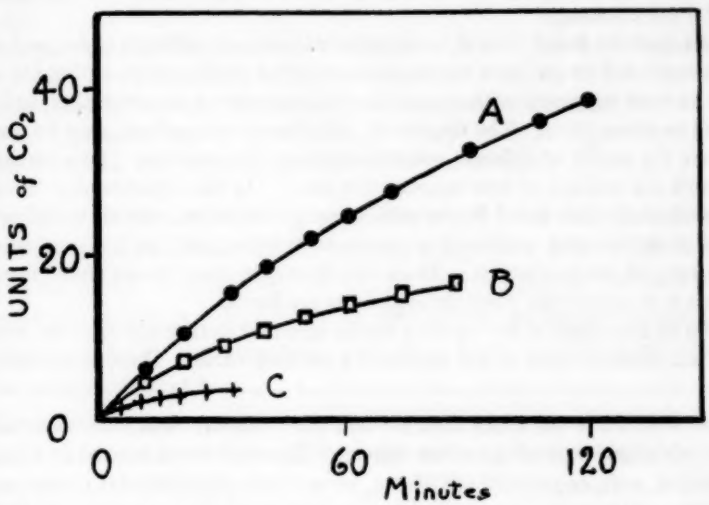

FIG. 1. The quantity of CO_2 produced by 0.1 gm. of pyrogallol, 1 cc. of H_2O_2, 44 cc. of water, and 10 cc. of

in Curve *A*, 0.0003 M; $CuCl_2$
" *B*, 0.0001 M; "
" *C*, 0.00001 M; "

The ordinates represent arbitrary units of CO_2 and the abscissæ minutes. Each curve is the average of three or more experiments.

days of continuous observation. The upper curves, therefore, are incomplete.

In order to compare and analyze curves of this sort the equations should be known. The attempt was made to fit the curves with the equations of a monomolecular and bimolecular chemical reaction. Although in some instances an approximate constant can be obtained, the correspondence is not very close, and these equations have very little significance in the present connection. However,

the curves can be fairly closely fitted by the general equation for the hyperbola $y = x/(a + bx)$. In all the experimental cases, when x/y is plotted against x the resulting figure is a straight line. There are individual deviations of slight extent but these are irregular in their occurrence and indicate that the experimental curves are fitted only approximately by the general equation $y = x/(a + bx)$. Even though the correspondence is only approximate, however, it is sufficiently close to permit the use of the equation in a purely empirical way for the comparison of the curves. In considering the present data the special equation may be used $a = t/(p + bt)$, where a is the amount of carbon dioxide produced after time, t, and p and b are constants.

The constants p and b must be determined for each different curve and when determined[4] will be an index to the characteristics of the curve, within the same limits as were suggested with respect to the accuracy of the general equation as applied to these cases. The constants, with these reservations, may be used to compare the action of different concentrations of the reagents. To evaluate the constants the method of least squares was used.[5] In the experimental curves we are particularly interested in the total amount of carbon dioxide which will be formed if the reaction is allowed to run to completion, and the rate, specially the initial rate, of the production. These two quantities may be calculated from the formula $a = t/(p + bt)$ when the constants are known.

From an inspection of the curves it can be seen that as time proceeds the amount of carbon dioxide, called a, will approach a limiting value. Then, in the formula,

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[4] One source of error arises from the fact that different values for the constants will be obtained, depending on how much of the entire curve is used as a basis of calculation, and, as pointed out above, some of the experimental curves are incomplete. This error is accounted for, at least partially, by using only the first half of the complete curve; *i.e.*, the curve up to the point where one-half of the total amount of the carbon dioxide has been produced. The total amount is observed in some cases and estimated in others.

[5] The method of least squares is probably the most accurate method of determining the value of p and b, although any two points may be selected on a curve and the constants obtained by substituting observed values of a and t. For these curves the method outlined by Mellor (1909, p. 327) has been followed. Using the general equation $y = y/(a + bx)$ and a large number of points the following equations determine the constants:

$$a = \frac{\Sigma\,(xy)\;\Sigma\,(x^2y^2) - \Sigma\,(x^2y)\;\Sigma\,(xy^2)}{\Sigma\,(y^2)\;\Sigma\,(x^2y^2) - (\Sigma\,(xy^2))^2}$$

and

$$b = \frac{\Sigma\,(xy)\;\Sigma\,(xy^2) - \Sigma\,(x^2y)\;\Sigma\,(y^2)}{(\Sigma\,(xy^2))^2 - \Sigma\,(x^2y^2)\;\Sigma\,(y^2)}.$$

allow t to become very large. Since p is constant, t, and also bt, will become so large that p may be neglected. Then $a_{(\lim)} = t/bt$. Cancelling t, $a_{(\lim)} = 1/b$. The limiting value of a and the total amount of carbon dioxide (expressed in arbitrary units of course) will therefore equal $1/b$. This furnishes a convenient method for comparing the total amount of the action under different conditions.

In considering the rate of the activity we may deal with either a derivative or a tangent depending on whether we are considering an equation or a curve. With the experimental curves the tangents may be ascertained by means of instruments, and the rate curves then plotted if desired. The initial rate may be observed directly.

With the equation the procedure is different. If $a = t/(p + bt)$ then $da/dt = p/(p + bt)^2$. By substituting values for t the value of da/dt, or the rate, may be plotted. Now let t become exceedingly small compared with p, and the differential equation approaches the value $da/dt_{(\lim)} = p/p^2$ or $1/p$. Therefore $1/p$ represents the initial rate of the production of carbon dioxide.

Applying these methods it is possible to get information concerning the concentration effects of the various reagents. Fig. 2 shows three curves obtained by plotting the values of $1/b$ against the concentrations of the reagents. Fig. 3 shows similar curves for the initial rates, as obtained from the equation and also from the tangents to the experimental curves.[6]

When the concentration of the pyrogallol or of the copper is varied we find that the limiting value of a varies as a constant fractional power of the concentration, or $a_{(\lim)} = C^k$. This relation has been found very frequently in biological work, and owing to its similarity to the adsorption isotherm it has caused many phenomena to be ascribed to adsorptive processes. The present case makes it plain that adsorption cannot always be called upon to explain every process where the effect is proportional to some fractional power of the concentration. For here we have nothing but a chemical system. Pyrogallol and hydrogen peroxide do not show colloidal properties, and it is very unlikely that copper chloride exists in anything but an ionic or molecular form. Hence if adsorption is responsible for the fractional

[6] The figures obtained by these two methods are almost identical in their general character, the differences being due to the fact that different units are employed in the calculations. This close correspondence between the two sets of curves is evidence of the soundness of the method of calculation used in determining $1/p$. The values calculated for $1/b$ may also be accepted with confidence, since the manner of derivation is the same.

power relation then it must be in a sense very different from that commonly employed.

With hydrogen peroxide a curious relation comes to light. When its concentration is varied and the logarithm of the concentration is

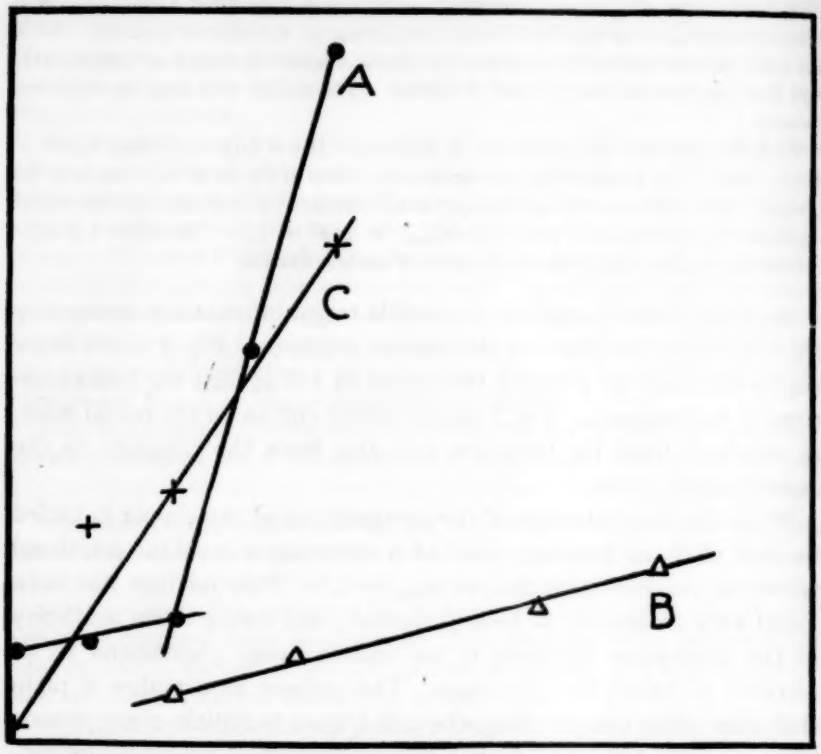

FIG. 2. Effect of concentration on the total amount of CO_2 produced. In all cases the ordinate is the logarithm of the amount of CO_2 as expressed by means of the logarithm of $1/b_{(\lim)}$ (see text). The abscissa is:

in Curve *A*, the logarithm of the concentration of $CuCl_2$;
" *B*, the logarithm of the concentration of H_2O_2 ÷ the logarithm of the amount of CO_2;
" *C*, the logarithm of the concentration of the pyrogallol.

plotted against the ratio of the logarithm of the concentration to the logarithm of the effect a straight line is obtained. Expressed as an equation $\log a = \log C/(m + n \log C)$. That this relation has been

previously observed may be shown by four instances where data from Snapper (1912), Dreyer and Walker (1914), Nothmann-Zuckerkandl (1912), and Plavec (1900), when plotted, give a straight line under similar conditions. However, the relation is unusual and no interpretation is available at present.

Fig. 2, Curve *A*, shows the effect of varying the concentration of

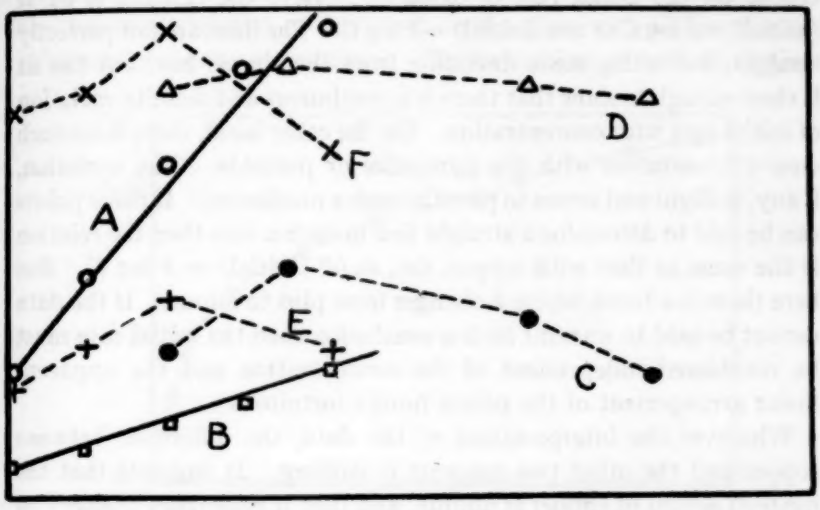

FIG. 3. Effect of concentration on the initial rate of reaction. In all cases the abscissa is the logarithm of the concentration. The ordinate in Curve *A* is the value of $1/p_{(lim)}$ for $CuCl_2$;

in Curve *B*, the value of tangents to the beginning of experimental curves of $CuCl_2$;

" *C*, the value of $1/p_{(lim)}$ for H_2O_2;

" *D*, the value of tangents with H_2O_2;

" *E*, the value of $1/p_{(lim)}$ for pyrogallol;

" *F*, the value of tangents with pyrogallol.

copper. It will be noticed that while in general the law $a_{(lim)} = C^k$ is obeyed, still, at 0.0001 M, there is a distinct break and the exponent k is different on each side. This is a situation which exists quite frequently in biological data. It might be maintained that at this critical concentration some change of phase relations occurs, such as the appearance of a new hydrate, dissociation product, or the like,

which would suddenly alter the effective concentration of the copper. While it is impossible to offer an adequate and detailed explanation of this phenomenon, nevertheless certain interesting possibilities are suggested, particularly with regard to phase relations in biological experiments.

Fig. 3, Curve *A*, shows the effect of changing the concentration of copper on the initial rate of oxidation. Here the relation is da/dt (initial) $= k \log C$ or tan (initial) $= k \log C$. The lines are not perfectly straight, indicating some deviation from the simple law, but the fit is close enough to show that there is a continuous and definite variation of initial rate with concentration. On the other hand, there is no such clear-cut variation with the pyrogallol or peroxide. The variation, if any, is slight and seems to pass through a maximum. If three points can be said to determine a straight line in such a case then the relation is the same as that with copper, *i.e.*, da/dt (initial) $= k \log C$. But here there is a break where k changes from plus to minus. If the data cannot be said to warrant such a conclusion then the initial rate must be considered independent of the concentration and the apparent linear arrangement of the points purely fortuitous.

Whatever the interpretation of the data, the difference between copper and the other two reagents is striking. It suggests that the mode of action of copper is unique, and that it undergoes changes, or takes part in reactions, in which the other two constituents are not involved.

IV.

After copper the first metal investigated was iron. With hydrogen peroxide and pyrogallol it causes a rapid evolution of carbon dioxide. Contrary to what might have been expected, iron was but slightly more effective than copper. This doubtless is due, however, to the conditions existing in this particular system. Under other circumstances iron is a much more effective oxidation catalyst than copper. At the same time it should be borne in mind that as a toxic agent copper is far more powerful than iron.

The form of the time curve with iron was substantially the same as that with copper. The concentration effect was not investigated. One phenomenon worth mentioning appeared in connection with the

experiments on iron. If several experiments were performed, using the same concentrations of all reagents, each experiment showed a larger production of carbon dioxide than the one preceding. This rendered it almost impossible to duplicate an experimental result. When the inside of the reaction chamber was coated with paraffin and the paraffin renewed occasionally, this difficulty vanished. The results became reproducible. Such a situation indicates that part of the iron was adsorbed or deposited on the glass wall of the tube and influenced subsequent reactions. No deposition of iron took place

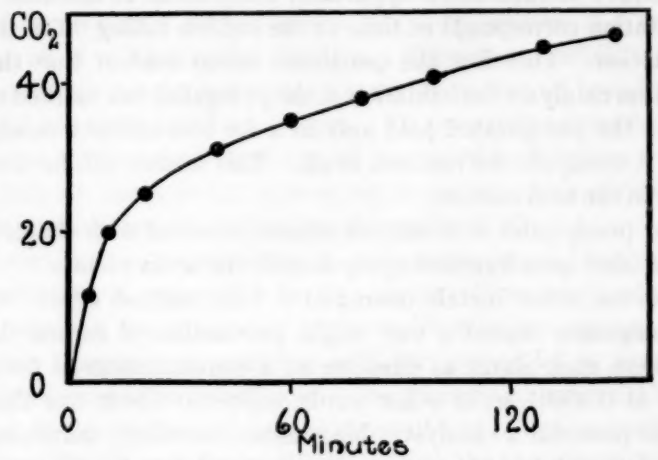

FIG. 4. Curve (average of three experiments) with $AuCl_3$, 0.01 M. The ordinate is the amount of CO_2 and the abscissa is time in minutes.

on the paraffin. Although no perceptible influence was ever exercised by the walls of the tube in the experiments on copper, paraffin was used as a safeguard.[7]

Several experiments were performed with silver nitrate and gold chloride. The action of these two elements being very similar only one, gold, will be discussed in detail. When the reaction with gold chloride starts, the production of carbon dioxide is relatively rapid (although not quite so rapid, for equivalent concentration, as with

[7] There are numerous instances recorded concerning the effect of the glass wall of the containing vessel on chemical reactions.

iron or copper). This period of great activity is followed by a much longer period of slight activity (see Fig. 4). The curve rises quite steeply for about 10 minutes, at the end of which time the rise becomes much more gradual. It appears as if there were two distinct curves, the first ending after about 10 minutes and merging into the second which continues for at least 2 hours. Examining the reagents it is found that the gold, which is introduced as a clear, yellow, solution of gold chloride, has precipitated as purple colloidal gold. If the reaction is followed in a test-tube it is observed that the precipitation begins after about 5 minutes and is apparently complete at 10 minutes. This precipitation corresponds in time to the sudden falling off in the rate of oxidation. Therefore the conclusion seems evident that the gold in solution catalyzes the oxidation of the pyrogallol to a marked extent, whereas the precipitated gold acts as a far less efficient catalyzer if indeed it catalyzes the reaction at all. This matter will be discussed further in the next section.

Silver precipitates in a manner almost identical with that of gold, and the same considerations apply as with the latter metal.

Of several other metals none had a very marked effect. Cobalt and manganese caused a very slight production of carbon dioxide. They were each about as effective at a concentration of 0.01 M as copper at 0.00001 M; in other words, copper is about one thousand times as powerful a catalyst. Magnesium, mercury, cadmium, zinc, tin, and nickel had no detectable effect whatever. Hydrogen, in hydrochloric acid, was likewise without effect. The metals investigated may therefore be arranged in the following manner:

Group I: *Catalysts* (in order of effectiveness), Fe, Cu, Au, Ag, Co, Mn.

Group II: *Non-Catalysts*, Mg, Hg, Cd, Zn, Sn, Ni, H.

The theoretical significance of this grouping will be discussed later.

V.

When we come to consider the probable mechanism of this oxidation system two facts stand out as of primary importance:

1. All the experiments were performed in an acid medium. Hopkins (1925) has found in studying the mechanism of oxidation by means of glutathione that there is a marked difference in the course of the

reaction depending on whether the medium is acid or alkaline, and Meyerhof (1923) maintains that oxygen transfer by sulfhydril groups occurs only in acid and best at pH 3 to 5. (On catalysis by Fe in this connection, *cf.* Harrison, 1924.) These and other instances make it apparent that a change from an acid to an alkaline medium may profoundly alter the mechanism of an oxidation. Therefore it should be borne in mind in the discussion of the present data that all conclusions apply to experiments performed in an acid solution and that very different results might be obtained with experiments performed in an alkaline solution.

2. The experimental curves do not follow the course of a monomolecular or of a bimolecular reaction. Since the possibility of a reaction of a higher order is remote, it is apparent that we have here a complex of reactions of different types. To construct a hypothetical arrangement which would duplicate all the experimental data would be very difficult, but a simple system can be set up which will give a general representation of what is found experimentally.

Let us consider the action of copper. There are two distinct phases to the oxidation of pyrogallol by this metal. First, the hydrogen peroxide must be decomposed in order to liberate the oxygen; and second, the oxygen must combine with the pyrogallol to produce carbon dioxide and other substances. Considerable research has been performed on the problem of the decomposition of hydrogen peroxide by metals. Bredig and Ikeda (1901) studied the decomposition of peroxide by platinum and considered that the action was due to the formation and reduction of an oxide of platinum. Oliveri-Mandalà (1920) found that a similar catalytic effect is produced by iridium, as had Bredig and Fortner (1904) with palladium. Berthelot (1901) found that silver forms a peroxide and afterward a superoxide with hydrogen peroxide, both of which decompose and liberate oxygen. Baeyer and Villiger (1901) started with the oxide Ag_2O and produced free oxygen with hydrogen peroxide. Colloidal silver was precipitated during the reaction. Hedges and Myers (1924) studied a periodic decomposition of hydrogen peroxide by silver, platinum, gold, and the enzyme catalase. These authors do not believe in the formation of an intermediate oxide.

In connection with the problem of "promoter action" the effect of

copper and iron, as well as other substances, has been extensively investigated. Bray and Livingston (1923) worked with bromine. Their opinion is that the hydrogen peroxide is decomposed by the bromine molecule which is thereby ionized and which is later restored to the molecular state. The ion is therefore the intermediate product. Von Bertalan (1920)[8] says: "Die Zerzetzung des Wasserstoffperoxyds durch Eisenionen verläuft als eine typische monomolare katalytische Reaktion." He considers the ion as the effective catalyst. Mummery (1913)[9] says: "The catalytic decomposition of hydrogen dioxide by iron salts may be ascribed to the formation of higher perhydrols which are to be regarded as derivatives of hydrogen trioxide." Bohnson (1921) says that with iron the intermediate product is a hydrated ferric peroxide or "ferric acid." It is of interest that his data relating concentration to velocity constant (he finds the reaction to be of the first order) show breaks in continuity of very much the same sort as the break in Fig. 2, Curve *A*, where copper is the catalyst. Both ferric chloride and ferric sulfate exhibit this phenomenon. Bohnson also found that the specific reaction velocity decreases with time owing to the hydrolysis of the catalyst, and that free acid retards the action. Bohnson and Robertson (1923) conclude that the reaction, with iron, is an oxidation of the ferric ion, Fe, to a peroxide, FeO_4, and subsequent reduction to the ionic form. They base their opinion on thermodynamic reasoning. Goard and Rideal (1924) state that the intermediate compound varies according to the acidity of the solution; that in neutral solution it is Fe_2O_5 and in acid solution it is H_2FeO_4. Similarly, Spitalsky and Petin (1924) ascribe the activity of iron to intermediate products which vary in composition with the hydrogen ion concentration. Robertson (1925) has summed up the discussion and has given an explanation of the so called promoter action of metals. For the simple combination of copper and hydrogen peroxide he suggests the following equations:

$$Cu + H_2O_2 \rightarrow CuO_2 + 2\,H$$

$$CuO_2 + H_2O_2 \rightarrow Cu + 2\,H_2O + O_2$$

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[8] von Bertalan (1920), p. 328.

[9] Mummery (1913), p. 889.

The CuO_2 is copper peroxide and when in acid solution may exist as H_2CuO_3.

In view of the above accumulation of opinion and evidence it seems permissible to accept Robertson's equations as the basis for the first step in the oxidation of pyrogallol, and to emphasize the following three points:

1. The metal functions in the ionic form.
2. There is always an intermediate product formed.
3. This intermediate product is a peroxide.

This system applies when there is simply a metal and hydrogen peroxide present. When an oxidizable substance is added it is necessary to alter the supposed course of the reaction. The fact that hydrogen peroxide will color guaiac in the presence of a metal salt has long been known. A recent study is that of Aloy and Valdigué (1923) on copper. Karczag (1921) has investigated the oxidation of dyestuffs by metal salts and hydrogen peroxide. Another investigation bearing on the present problem is that of Bredig and Ikeda (1901), who found that many organic substances, including pyrogallol, "poisoned" the reaction between the metal and the peroxide.

When an organic substance such as pyrogallol is oxidized by means of this system one of two things must happen. Either the oxygen which is liberated from the hydrogen peroxide unites with the pyrogallol, or the oxygen is transferred directly from the metallic peroxide without any separate existence. The latter supposition is more probable and is entirely in line with current theories of oxidation. If we accept it then we must notice two facts:

1. The second stage of Robertson's reactions is altered because the oxygen in the CuO_2 does not escape as molecular oxygen but is transferred directly to the pyrogallol.

2. If this transfer released the ionic copper in its original state then the copper could go through the same cycle again and the reaction would continue until either the pyrogallol or the hydrogen peroxide was exhausted. But we know from experiments with low concentrations of copper that this is not the case. For instance, if we use 0.00001 M copper the production of carbon dioxide soon stops. If we then add more peroxide or pyrogallol there is no effect; but if we add more copper the oxidation begins again. This fact demonstrates

conclusively that the copper is steadily removed from the active state. The removal may take place in various ways but the simplest assumption is that the metal is bound up irreversibly with the pyrogallol. That some such process occurs is suggested by the results of Bredig and Ikeda (1901) on the "poisoning" effect of pyrogallol.

Let us call pyrogallol PC. Then

$$CuO_2 + PC \rightarrow CuP + CO_2$$

The carbon dioxide is measured and the inactive, or bound, copper remains in the solution. But we must complete the original equation. This we may do and write the entire scheme:

(1) $$Cu + H_2O_2 \rightarrow CuO_2 + 2\,H$$

(2) $$2\,H + H_2O_2 \rightarrow 2\,H_2O$$

(3) $$CuO_2 + PC \rightarrow CuP + CO_2.$$

Of course this scheme may differ in detail from what actually takes place. The real reactions are doubtless more complicated than is indicated here. But it is possible to go a certain distance toward duplicating the experimental results by means of the theoretical equations. Considering reactions (1) and (2) it is evident that there is a bimolecular reaction between the copper and the hydrogen peroxide. But there are two molecules of hydrogen peroxide decomposed for every molecule of copper peroxide formed, owing to the reduction of the second molecule of hydrogen peroxide by the free hydrogen. Using the ordinary notation, if a is the initial concentration of copper, b the initial concentration of the hydrogen peroxide, and y the amount of copper peroxide formed after time t, then the rate of the reaction is proportional to the copper and hydrogen peroxide present. Of the latter, part is used in step (1) to form CuO_2 and H, and part in step (2) to form H_2O. These two parts are equal. Then we may write $dy/dt = K_1 (a - y) (b - 2y)$. This equation may be integrated and expressed in the following form:

$$y = \frac{a\,b\,(1 - e^{K_1 (a - b) t})}{b - 2\,a\,e^{K_1 (a - b) t}}.$$

If x is the amount of carbon dioxide formed after time t, and c is the concentration of the pyrogallol, then x is proportional to the amount of pyrogallol and the amount of CuO_2 (or y) present after t units of time. This amount of CuO_2 is equal to the difference between the quantity produced from a and b and the quantity which has been transformed into x. Then $dx/dt = K_2 (y - x) (c - x)$, or

$$x = \frac{y\,c\,(1 - e^{K_2(y-c)t})}{c - y\,e^{K_2(y-c)t}}.$$

Since y is a variable quantity its value must be determined for every value of t and substituted in the above equation.

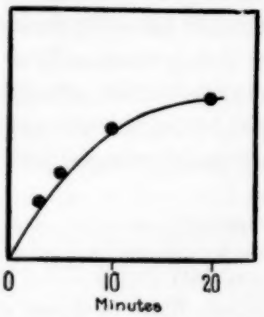

Fig. 5. The solid line represents a typical experimental curve ($CuCl_2$ 0.001 M H_2O_2 1 cc., pyrogallol 0.001 gm.). The points are obtained by means of the calculation outlined in the text whereby two bimolecular reactions are assumed. In this case $a = 0.5$, $b = 2$, c = 0.08, $K_1 = 0.2$, and $K_2 = 0.2$.

By the use of these equations the experimental curves may be approximately duplicated. Fig. 5 shows an example of an experimental curve selected at random and fairly closely fitted. The fit could be made much closer by more exact selection of the constants. This method of curve duplication does not afford absolute proof that the equations underlying the calculations are precisely those which underlie the experimental results. However, they must be fairly close to the truth. That the actual state of affairs is more complicated than has been suggested here is shown by one fact. In the calculated system the effect varies directly with the concentration, not as a fractional power thereof. In order to bring the calculated results into accordance with the experimental results it would be necessary

to assume further complications such as side reactions, opposing reactions, *etc.* But the experimental data do not clearly indicate the precise nature of these additional reactions.

The definite conclusions which may be drawn are as follows: The oxidation of pyrogallol is due to the decomposition of the hydrogen peroxide by the copper ion, with the subsequent peroxidation of the copper. The copper peroxide then transfers its oxygen to the pyrogallol which is broken down into carbon dioxide and other products, including some form of combined copper.[10]

This system coincides with the Engler theory of respiration, in which there is an autoxidizable substance, a peroxide, and a catalyst. Atmospheric oxygen is absorbed and transferred by means of the peroxidase (catalyst) through the peroxide from the autoxidizable substance to the substance which is eventually to be oxidized. Here, of course, we start with a peroxide but the principle is the same. Wieland (1921) takes the view that hydrogen, not oxygen, is transferred, but he states that with metals and hydrogen peroxide metallic peroxides

[10] The question of the atmospheric oxidation of pyrogallol and the nature of the products when the reaction is catalyzed by a metal has received attention from chemists. The two best known oxidation products are carbon dioxide and purpurogallin. The latter is specially likely to appear when iron is present. Salts of iron as well as of other metals may combine directly with pyrogallol, the reaction usually showing a characteristic color. Thus Jacquemin (1873) found that ferrous sulfate gives a blue color and ferric sulfate a red one.

de Clermont and Chautard (1882) got purpurogallin from pyrogallol in acid solution with $AgNO_3$ and $KMnO_4$. Wolff (1908) used ferrocyanide with hydrogen peroxide and obtained purpurogallin. Smirnow (1925) determined the effect of peroxidases by measuring the formation of purpurogallin, and found that manganese and iron catalyze the reaction although he does not consider that these two metals are necessary to a peroxidase. He quotes André (André, G., 1924, Chimie végétale, i, 210) to the effect that pyrogallol oxidizes according to the equation: $4\ C_6H_6O_3 + 9\ O \rightarrow C_{20}H_{16}O_9 + 4\ CO_2 + 4\ H_2O$ where $C_{20}H_{16}O_9$ represents purpurogallin. He also quotes Willstätter and Stoll (Willstätter, R., and Stoll, A., 1918, *Ann. Chem.*, cdxvi, 62), who state that $2\ C_6H_6O_3 + O_3 \rightarrow C_{11}H_8O_5 + CO_2 + H_2O$. The latter formula ($C_{11}H_8O_5$) is given as the correct formula for purpurogallin in Beilstein, F., 1923, Handbuch der organischen Chemie, 4th edition, vi, 1072. In the experiments here recorded the concentrations of the reagents were too low for noticeable quantities of purpurogallin to be produced.

are formed. He therefore agrees in essentials with the intermediate peroxide theory.

VI.

Throughout the preceding discussion the assumption has been made that the metals act in the ionic form. This assumption is based on the results of Bohnson, Robertson, etc., according to whom the metals (in decomposing hydrogen peroxide) take part in ordinary chemical reactions. There is no reason to suppose that there is any change in the state of iron or copper when pyrogallol is present. Nevertheless there is evidence to show that certain metals in the colloidal state can also decompose hydrogen peroxide. Certain metals, furthermore, are precipitated in the colloidal form by pyrogallol. Bredig and Reinders (1901) decomposed hydrogen peroxide with colloidal gold, as did Galecki (1925). Bredig and Ikeda (1901) used colloidal platinum, Duclaux (1923) used colloidal iron, and Zenghelis and Papaconstantinos (1920) used colloidal rhodium. As to the effect of pyrogallol, in addition to calling attention to the reduction of silver bromide by pyrogallol in photography, reference may be made to Garbowski (1903), who precipitated gold, platinum, and silver, and to Henrich (1903), who precipitated gold, platinum, silver, and mercury with pyrogallol.

We have a very clear example of the different effect of ionic and colloidal metal in the experiments performed on gold which were outlined in a previous section. In these experiments we can watch the rate of oxidation change while the colloidal gold is being precipitated. The first few readings are obtained with ionic gold and show a rapid action. After the gold has been precipitated the action still continues but at a greatly reduced rate. There exist at this point two possibilities: either the gold is all precipitated and the slow action is due to the colloidal metal, or not all the gold is precipitated, the colloidal metal has no effect, and the slow rate is due to a very much reduced quantity of the ionic metal. To throw light on this matter a standard quantity of gold chloride was added in a test-tube to the usual amount of hydrogen peroxide and pyrogallol and after the gold had been precipitated the entire mixture was dialyzed for a week.

Then the colloidal gold was recovered, added to more hydrogen peroxide, and pyrogallol and the resulting action tested in the respiration machine. There was no detectable production of carbon dioxide. The conclusion follows that colloidal gold does not catalyze the oxidation of pyrogallol in measurable quantities, despite the fact that it decomposes hydrogen peroxide.

The question arises why colloidal gold does not oxidize pyrogallol. It will be remembered that to account for the action of copper it was assumed that the copper forms a peroxide which carries the oxygen from the hydrogen peroxide to the pyrogallol. It does not liberate molecular oxygen which then, of itself, combines with the pyrogallol. The formation of the intermediate peroxide is essential. Now it is clear that while ionic gold can react to produce a gold peroxide, the metal in the colloidal form cannot do so. Therefore as the gold in solution becomes aggregated in solid particles the concentration of the effective gold is reduced. That the ions are not completely eliminated and that all the gold is not changed into the colloidal form (as is demonstrated by the fact that some oxidation persists) is probably due to the fact that the solution is acid. That the degree of dispersion, size of particles, and general properties of colloidal metals are dependent to a large degree on the acidity of the medium has been shown by numerous investigators. The presence of the pyrogallol doubtless has considerable influence and may tend to prevent the complete precipitation of the gold in solution.

Obviously the mode of action of the metal in the colloidal state is very different from the mode of action in the ionic form. As the present case shows, ionic gold will catalyze the oxidation of pyrogallol while colloidal gold will not. But the latter will decompose hydrogen peroxide as well as the former. The decomposition, however, must be due to an entirely different mechanism in the two cases. The intermediate peroxide theory, which is very satisfactory with the metal in solution, offers no explanation whatever for the action of the colloidal metal since the colloid cannot be expected to enter into simple stoichiometric relations with the other constituents, including the formation of metallic peroxides. If, as has been assumed, the oxidation of pyrogallol depends on the presence of such peroxides, it is quite evident

why colloidal gold can decompose hydrogen peroxide but fails to oxidize pyrogallol.

VII.

We are now in a position to make further comparison between the several metals which have been tested. A division has already been made between the effective catalysts, copper, iron, gold, silver, cobalt, and manganese, and the non-effective metals which include all the others tried. Among the first four there are marked differences depending on the ease with which the colloidal metal is precipitated. As a check on this point test-tube experiments were made which showed that gold and silver precipitated very easily, copper very slowly (a matter of days), and iron not at all. Iron is therefore the most powerful catalyst because it readily forms a peroxide and has the least tendency to be precipitated in the colloidal form by hydrogen peroxide and pyrogallol. Gold and silver are effective catalysts (though not so effective as iron and copper), but are prevented from exercising this function because they are so easily precipitated. Mercury, zinc, and the others, are ineffective because they do not form intermediate peroxides.

Herein may lie the reason why iron, and secondarily copper, are almost universally found in living cells as the metals which catalyze respiration. The assumption is legitimate that the cell contains organic peroxides similar to hydrogen peroxide and reducing (easily oxidizable) substances like pyrogallol. If this is true, and there is no strong evidence against it, then iron and copper are probably the only two metals which could effectively catalyze the oxidations in the cell. For metals like cobalt and manganese would be too weak in their action, mercury, zinc, etc., would not carry oxygen at all, and gold or silver would pass immediately into the ineffective colloidal state. Therefore iron and copper best fulfil the requirements for a metallic catalyst.

One final theoretical aspect of this question deserves brief consideration. If we examine the periodic table of the elements as revised to conform to the most recent ideas in physical chemistry, we find that all the active elements here investigated, *i.e.* iron, copper,

gold, silver, cobalt, and manganese will be found close together in the table. Below is a reproduction of a portion of the table showing the arrangement of electrons in the atoms of the elements (according to Langmuir), found in Taylor's Treatise on physical chemistry (1924):[11]

	IIIa	IIIb	IVa
Group VII	Mn	—	—
Group VIII	Fe	Ru	Os
	Co	Rh	Ir
	Ni	Pd	Pt
Group I	Cu	Ag	Au
Group II	Zn	Cd	Hg

The groups refer to the standard groups in the periodic table. (The numerals at the head of the columns refer to the number of shells of electrons.) Thus we see that Group I, which includes copper, silver, and gold, has an outer shell of one electron, and Group VIII has an outer shell which lacks completeness by one electron. It is in these two groups that the effective elements Fe, Cu, Ag, Au, and Ru, Rh, Pd, Os, Ir, and Pt, which are probably also effective, occur. The only exceptions are manganese which is in Group VII, and nickel which is in Group VIII but is not effective. In general the situation is striking. Those elements which are effective oxidation catalysts have their outer shell either composed of just one electron or lacking just one electron. Furthermore, as the number of shells increases the greater becomes the tendency for the element to pass into the colloidal state with hydrogen peroxide and pyrogallol. It is impossible to draw any conclusions at present from these facts, but they are very suggestive and indicate the possibility of future theoretical developments.

SUMMARY.

1. When iron and copper are allowed to act on hydrogen peroxide and pyrogallol, enough carbon dioxide is produced to be readily measured.

[11] Taylor (1924), p. 1062.

2. The curve of the production of carbon dioxide may be fitted by an empirical equation, by the use of which the initial rate and the total amount of the oxidation may be determined.

3. The effect of the concentration of the reagents is different in each case, the effect varying as a fractional power of the copper and pyrogallol concentrations and as a logarithmic function of the hydrogen peroxide concentration.

4. When gold or silver is used the rate changes suddenly during the course of the reaction due to the precipitation of colloidal metal.

5. Mercury, cadmium, zinc, tin, and some other metals have no effect.

6. A theoretical set of equations is assumed to account for the action of the metals.

7. The metals are assumed to act by means of the formation of intermediate peroxides.

8. Experiments on the action of gold indicate that the metals are active in the ionic and not in the colloidal state.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

I. THEORETICAL.*

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The attempts to explain a part of the behavior of living cells or tissues on the assumption that they act as simple ampholytes, while fairly satisfactory and suggestive, must be considered only as a first suggestion. Those who employ this concept admit that living cells are more complicated systems than simple proteins, so that clear-cut results from studying them from such a point of view are not to be expected. Certain recent researches cannot, however, be easily correlated on the basis of mere lack of "clear-cutness." For example, Robbins (1), from the staining reactions and water absorption of potato tuber, has shown that it acts as an ampholyte with an isoelectric point at a pH of about 6. However, Cohn, Gross, and Johnson (2) have found that the typical potato protein, tuberin, obtained by acid precipitation of potato juice, has an isoelectric point at a pH of about 4.

Winslow, Falk, and Caulfield (3) have studied the electrophoretic behavior of the organism *Bacillus cereus* over a wide pH range, and while, in the main, the curve obtained may be explained on the basis of a simple Donnan equilibrium, there is a comparatively wide pH range through which such an explanation cannot hold.

Certain unicellular organisms seem to show little tendency to retain either acid or basic dye, through a comparatively wide pH range, in place of through only a narrow range as might be expected to be characteristic of the isoelectric behavior of a simple ampholyte (4). Other organisms show no point where combination with one

* Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 115.

or the other type of dye does not seem to take place to a considerable extent.

It is the purpose of this paper to examine the probable behavior of a system of two amphoteric substances between which mutual combination may take place under proper conditions, and to show that, by employing the considerations involved, it is much easier to explain much of the physical and chemical behavior of living tissues than it is by using the concept of a simple ampholyte. In the two following papers experimental evidence is adduced, from a study of certain simple systems of two ampholytes, in support of this idea. It may, however, be pointed out that living cells are by no means as simple as this above concept would seem to indicate. Its justification lies in the fact that, by sacrificing only very little of the simplicity of treatment which suffices for consideration of simple ampholytes, one gains greatly in comprehensiveness.

Concept of a Conjugate Protein.

Consider an aqueous solution of two amphoteric substances, HAOH with ionization constants K_a and K_b and an isoelectric point at a hydrogen ion concentration I, and HBOH with corresponding constants K'_a and K'_b and an isoelectric point at I'. Suppose I is larger, *i.e.* more acid, than I'.

In solutions whose hydrogen ion concentration is appreciably greater than I both components will act as bases and tend to retain acids. When the hydrogen ion concentration is appreciably less than I' both components will act as acids and tend to retain bases. In solutions whose hydrogen ion concentration lies between I and I', however, one component will act as a base and the other as an acid, and there will be a tendency toward mutual combination, resulting in a decreased retention of acid or basic reagent. This pH range, from I to I', is the range of mutual combination; and the stability of the system, so far as its behavior as a distinct individual is concerned, depends among other things on the magnitude of this range. This might explain, for example, why certain conjugated proteins, such as lecithoproteins, though they are thought to exist, have not been definitely isolated, while others such as nucleoproteins or phosphoproteins can be easily obtained.

Apparent Isoelectric Behavior.

In a system of two ampholytes we have the following equilibria:

$$\overset{+}{H} + AO\overset{-}{H} \rightleftarrows HAOH \rightleftarrows H\overset{+}{A} + \overset{-}{OH} \text{ with constants } K_a, K_b, \text{ and } I;$$

and

$$\overset{+}{H} + BO\overset{-}{H} \rightleftarrows HBOH \rightleftarrows H\overset{+}{B} + \overset{-}{OH} \text{ with constants } K'_a, K'_b, \text{ and } I'.$$

If HAOH is the more strongly acidic, there will be an isoelectric point at the pH at which

$$(AO\overset{-}{H}) + (BO\overset{-}{H}) = (H\overset{+}{A}) + (H\overset{+}{B})$$

The value of the hydrogen ion concentration corresponding to this point is obtained by expressing the above quantities in terms of $(\overset{+}{H})$ and the various constants and solving. We obtain

$$(\overset{+}{H}) = \sqrt{K_w \frac{K_a (HAOH) + K'_a (HBOH)}{K_b (HAOH) + K'_b (HBOH)}} \qquad (1)$$

The isoelectric point is not exactly the point of maximum mutual combination. The latter point may be expected to be governed by the condition

$$(AO\overset{-}{H}) = (H\overset{+}{B})$$

and will occur at a hydrogen ion concentration obtained from the following expression:

$$(\overset{+}{H}) = \sqrt{K_w \frac{K_a (HAOH)}{K'_b (HBOH)}}$$

The two points, though not identical, will lie very close together; and the simple expression may be used for calculating the isoelectric point of the system.

Electrophoretic Behavior.

From the amphoteric equilibria given above we can, by applying the mass-action law and differentiating with respect to the logarithm

of the hydroxide ion concentration, obtain the following four equations:

$$\frac{d(\mathrm{A\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})} = \frac{K_a\ (\mathrm{HAOH})\ (\mathrm{\bar{O}H})}{K_w}. \tag{2}$$

$$\frac{d(\mathrm{\overset{+}{H}A})}{d\ ln(\mathrm{\bar{O}H})} = -\frac{K_b\ (\mathrm{HAOH})}{(\mathrm{\bar{O}H})}. \tag{3}$$

$$\frac{d(\mathrm{B\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})} = \frac{K'_a\ (\mathrm{HBOH})\ (\mathrm{OH})}{K_w}. \tag{4}$$

$$\frac{d(\mathrm{\overset{+}{H}B})}{d\ ln(\mathrm{\bar{O}H})} = -\frac{K'_b\ (\mathrm{HBOH})}{(\mathrm{\bar{O}H})}. \tag{5}$$

from which

$$\frac{d(\mathrm{A\bar{O}H})}{-\ d(\mathrm{\overset{+}{H}B})} = \frac{K_a\ (\mathrm{HAOH})\ (\mathrm{\bar{O}H})^2}{K'_b\ (\mathrm{HBOH})\ K_w}$$

or, since

$$(\mathrm{\bar{O}H}) = K_w/(\mathrm{\overset{+}{H}}),$$

$$d(\mathrm{A\bar{O}H}) = -\frac{K_a\ (\mathrm{HAOH}\ (\mathrm{\bar{O}H})}{K'_b\ (\mathrm{HBOH})\ (\mathrm{\overset{+}{H}})}\ d(\mathrm{\overset{+}{H}B})$$

Either an increase in $(\mathrm{A\bar{O}H})$ or a decrease in $(\mathrm{\overset{+}{H}B})$ will increase the resultant negative charge on the micella, and thus increase the velocity toward the anode in a constant electric field. At the isoelectric point, as the hydroxide ion concentration is increased, $\frac{d(\mathrm{A\bar{O}H})}{d\ ln(\mathrm{\bar{O}H})}$ is of the same order of magnitude as $\frac{-\ d(\mathrm{\overset{+}{H}B})}{d\ ln(\mathrm{\bar{O}H})}$ but the former increases with increasing alkalinity while the latter decreases (equations (3) and (4)). Through the pH range I to I', the value of $-d(\mathrm{\overset{+}{H}A})$

is small compared to that of $d(\overline{AOH})$, and $d(\overline{BOH})$ is small compared to $-d(\overset{+}{HB})$. As a result there will be a rather rapid increase in negative charge through a certain range, passing through the isoelectric point, until nearly all the HAOH is ionized to $\overline{AOH}$, which ionization takes place increasingly rapidly as $(\overline{OH})$ is increased. When such a condition is reached, the only possible significant increase in negative charge before the point I' is reached is from $-d(\overset{+}{HB})$, which has less and less effect on the magnitude of the charge as $(\overset{+}{HB})$ becomes smaller, *i.e.* as $(\overline{OH})$ is increased (equation (4)). This means that through a certain pH range the negative charge will remain nearly constant. When I' is reached, however, we have $\frac{-d(\overset{+}{HB})}{d(\overline{BOH})} = 1$, and from this point on $\frac{d(\overline{BOH})}{d\,ln(\overline{OH})}$ is the predominant factor. Its value increases with $(\overline{OH})$, and the negative charge again begins to increase more rapidly, and continues until the HBOH is completely ionized. From this point on, since we are now on the alkaline side of the isoelectric points of both components and they are thus both in the same ionic state, we may expect the curve to be the same as would be predicted by the application of the Donnan equilibrium to a simple ampholyte.

A similar condition would prevail on the acid side of the isoelectric point of the system as the hydrogen ion concentration is increased.

DISCUSSION.

It may be well to cite some of the observations which originally led to the more definite formulation of the concept of a mixed amphoteric system.

In connection with certain bacteriological problems the staining reactions of a large number of organisms have been studied by the author (4). Certain of the typical curves are given in Fig. 1.[1] Ab-

[1] Bacterial cells furnish a very satisfactory material to study. The individual cell as a system can be easily observed, thin smears can be obtained fairly free from débris, and equilibrium can be quickly reached. We found little difference in results between buffering for several minutes and for as long as 150 hours. Gern-

scissæ are pH values and ordinates are arbitrary functions of the intensity of retained color. Values for the latter were obtained by repeated comparison of slides under the microscope, and are there-

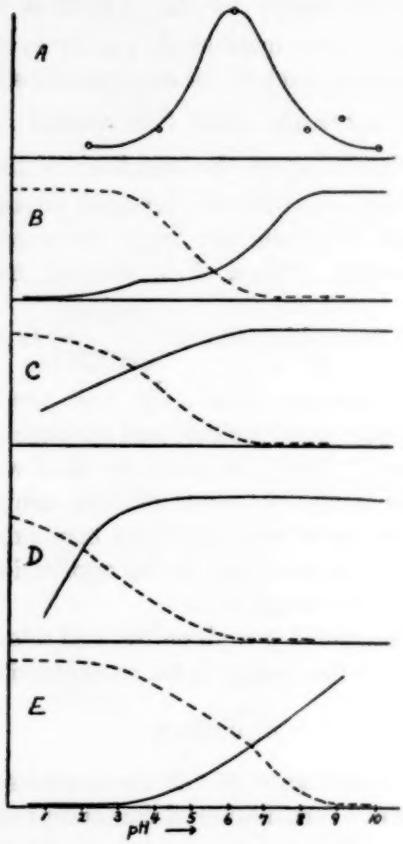

FIG. 1. For explanation see text. Broken lines represent behavior toward acid fuchsin, unbroken lines (except Curve A) behavior toward gentian violet.

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gross, along the same line (6), states that hide powder in small quantities, unhardened by formaldehyde, reaches complete reversible equilibrium with acid solutions in 2 minutes. Our final technique consisted in preparing thin smears on microscope slides, staining with "carbol gentian violet" or "carbol acid fuchsin" solution, "fixing" by treatment with buffer solution, and finally decolorizing with acetone. Slides of any series were then repeatedly compared under a microscope.

fore only qualitative. They are, however, comparative and the curves represent the behavior of the organism. The intensity plotting has been conservative and quantitative methods would, we feel sure, merely accentuate the contrast between the greater and smaller dye retention.

In Curve A of this figure the buffer ratios for the organism *Bacillus coli*, obtained by Falk and Shaughnessy (5) are plotted just above the color curve for the same organism (Curve B).[2] The results are suggestive, showing that the buffering power of this organism shows itself over a wide pH range with a maximum near the isoelectric point of the system as determined by minimum dye retention.

The curves in this figure are typical of classes of bacteria. A fair number of organisms have their point of least color retention around a pH of about 3 (Curve D), another large class has a corresponding point around a pH of 5 to 6 (Curve B), and there are a few intermediate (Curve C).

Other types of cells were studied, such as red blood cells from both human blood (Curve E) and sheep blood. In the case of the former the smears were made from whole blood and the isoelectric point of such a system lies a little below a pH of 7. The sheep cells used were washed cells and showed a corresponding point at a pH very near 7.

Such a system as *Bacillus coli* (Curve B) exhibits a range through which little dye is retained—either acid or basic—suggesting a considerable stability of the system as a chemical individual, while the system *Bacillus dysenteriæ* Shiga (Curve C), even at its isoelectric point, still retains fairly strongly both acid and basic dyes, showing that there is still a fair concentration of both cation and anion in the system, and that either actual combination between these two is limited, or that the combination is comparatively unstable.

The condition of maximum combination between the two components of a system of two ampholytes need not mean a condition of much combination. When $(\overline{AOH}) = (\overset{+}{HB})$, though this is the optimum condition for combination, the system may act as a majority

[2] In plotting Curve A the pH range given in the table by Falk and Shaughnessy is plotted at its lowest value, thus where they give the range 6–7, it is plotted as 6.

of ionogens and remain largely in ionic form. On the other hand it may act in a manner analogous to such salts as lead acetate, mercury salts, etc., and the two ions may almost entirely combine. The extent of this combination will determine the behavior toward anions and cations. In systems in which the two components are largely combined at the isoelectric point we may have behavior simulating a simple ampholyte in that there will be a pH range through which no appreciable combination with added cation or anion takes place.

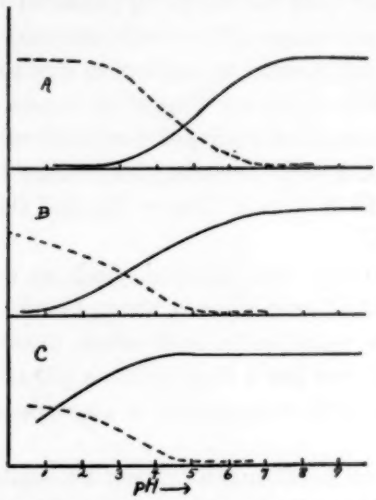

FIG. 2. Effect of oxidation on the behavior of *Bacillus typhosus* toward acid fuchsin (broken line) and gentian violet (unbroken line). Curve A—original organism, Curve B treated with N/50 iodine, Curve C treated with N/50 potassium dichromate.

On the other hand, we may have systems in which even at the isoelectric point there is still a fair concentration of both $(\overline{AOH})$ and $(\overset{+}{HB})$ and thus there will be no point at which added anions and cations will not be appreciably bound.

There are at least two ways in which the isoelectric point of a mixed system or of a simple ampholyte may be changed. The system may be transformed into a new mixed system either by altering the relative amounts of the components or by adding another component,

or the acid or basic properties may be altered by oxidation or reduction.

An example of the first effect is reported by Gerngross (7) who found that the electrophoretic isoelectric point of gelatin was changed from a pH of 4.75 to 4.3 by treatment with formaldehyde.

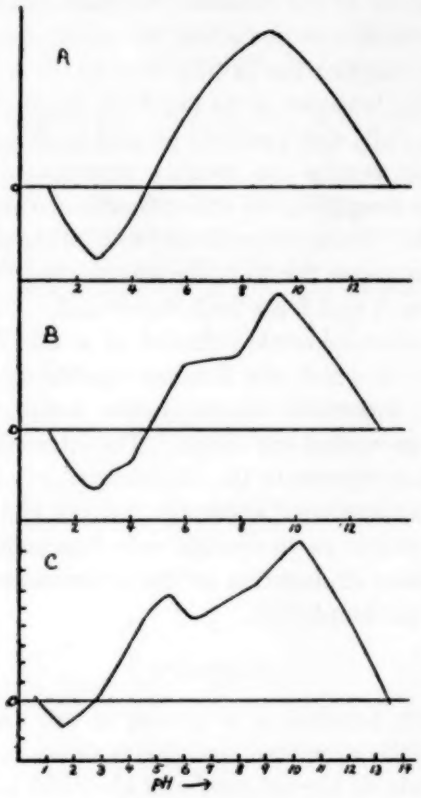

FIG. 3.

Change in isoelectric point by oxidation has been studied by the author (4), and certain results are shown in Fig. 2. The magnitude of the change depends on the degree of oxidation. A mild oxidizing agent such as iodine (Curve B) renders the system more acidic than it was originally (Curve A), but such an oxidizing agent as potassium

bichromate produces a distinctly greater shift in the same direction (Curve C).[3]

While reducing agents do not seem to have any effect on the original organism, the effect produced by oxidation has been repeatedly reversed by treatment with stannous chloride. This behavior is, of course, analogous to the behavior of practically all substances in the effect of oxidation or reduction on acidic strength.

An interesting application of the concept of a mixed system is the electrophoretic behavior of the organism *Bacillus cereus* as worked out by Winslow, Falk, and Caulfield (3) and by Winslow and Shaughnessy (8). Their results are roughly represented by Curve C of Fig. 3. Abscissæ are pH values and ordinates are migration velocities in an electric field. These are measured toward the anode on that portion of the curve above the zero line and toward the cathode below this line. Curves A and B are both theoretical. Curve A represents the theoretical electrophoretic behavior of a micella composed of a single ampholyte to which the Donnan equilibrium applies. Curve B represents the theoretical electrophoretic behavior of a system of two ampholytes as worked out above. The intersection of the curves with the zero line represents the isopotential point.

In all the cases mentioned above the concept of a mixed system of ampholytes, so simple as to contain only two components, offers a much more obvious explanation of the experimental facts than the concept of a simple ampholyte.

SUMMARY.

The amphoteric behavior of a system of two amphoteric components is theoretically examined; and this is shown to correspond more nearly with certain of the physical and chemical behaviors of living tissues than does the concept of a simple ampholyte.

[3] Oxidizing agents were incorporated in the buffer solutions in N/50 concentrations.[1] A goodly number of oxidizing agents were studied, and, in general, they could be arranged in a series on the basis of the magnitude of their effect on the organisms, which series was roughly the same as arrangement on the basis of oxidizing potential. The increase in acid properties upon treatment with an oxidizing agent, illustrated in Fig. 2, was noted in all organisms studied.

It is a pleasure to acknowledge the criticism and suggestions of Dr. A. L. Raymond in the preparation of the manuscript of this and the three following papers.

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AMPHOTERIC BEHAVIOR OF COMPLEX SYSTEMS.

II. Titration of Sulfanilic Acid-Glycine Mixtures.*

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The considerations presented in the preceding paper were developed as an attempt to explain results obtained in studying living cells (1). It is desirable to test them on much simpler systems. Michaelis and Davidsohn (2), in some flocculation experiments, obtained data which support the above ideas in a qualitative way. They state that, if two amphoteric colloids are mixed, a combination may precipitate out whose flocculation optimum lies between their respective isoelectric points. Thus with nucleic acid and denatured serum albumin, with isoelectric points of 2×10^{-1} and 4×10^{-6} respectively, there is a combination with optimum flocculation at a hydrogen ion concentration of 1.6×10^{-4}.

It is the purpose of this and the following paper to test portions of the theory in a more quantitative manner, and in this paper the mutual action of two ampholytes, glycine and sulfanilic acid, is studied by means of titration over a wide pH range. The latter substance was chosen to correspond to the strongly acid nucleic acid in living matter. That it is amphoteric, with a measurable basic ionization constant, is shown in the fourth paper of the series. In fact it has an isoelectric point at a distinctly higher pH, 1.25, than that (0.7) of nucleic acid (2). The glycine used was a preparation from the Eastman Kodak Company; and the sulfanilic acid was a Merck C.P. grade, which was reprecipitated from alkaline solution by hydrochloric acid, washed, and dried.

*Contributions from the Gates Chemical Laboratory, California Institute of Technology, No. 116.

Method.

An ordinary potentiometer set up sensitive to 0.25 millivolt, was employed. The hydrogen electrodes were of the type described by Bailey (3). A saturated calomel electrode (4) was used in connection with a saturated KCl bridge.

A series of solutions of hydrochloric acid and carbon dioxide-free sodium hydroxide was made up, each solution of definite known con-

TABLE I.

HCl				NaOH			cOH
N	E.M.F.	pH	cH	N	E.M.F.	pH	
	mv.				mv.		
.001	426.	3.00	.001	.004	924.5	11.425	.00266
.003	399.	2.533	.00293	.009	943.	11.74	.0055
.006	380.	2.217	.00607	.015	958.	12.00	.010
.009	370.	2.050	.00891	.025	968.5	12.175	.015
.012	363.	1.933	.0117	.040	981.7	12.390	.0246
.018	353.	1.760	.0174	.060	990.3	12.540	.0347
.025	345.5	1.625	.0237	.080	997.5	12.660	.0458
.040	333.5	1.425	.0376	.100	1001.5	12.730	.0538
.060	323.	1.250	.0562	.130	1008.5	12.860	.0720
.090	313.	1.083	.0826	.180	1014.8	12.960	.0916
.120	305.	0.950	.1122	.250	1024.	13.117	.1310
.150	300.5	0.875	.1334	.3511	1030.	13.217	.165
.200	292.5	0.742	.181				
.250	287.8	0.663	.217				
.320	282.	0.56	.2754				

centration. Points on the titration curve were obtained by introducing 15 cc. of one of these solutions into a small glass-stoppered bottle with 10 cc. of a standard solution of the substance to be titrated, and the equilibrium hydrogen ion concentration of the resulting mixture determined. The difference between the normality of the acid or base diluted with 10 cc. of water and that diluted with 10 cc. of the glycine or the sulfanilic acid will give the amount neutralized. The original normality, N, is known and the latter can be obtained from the measured cH if we know the degree of ionization, *i.e.* the normality is equal to cH/α, where α is the degree of ionization, which value

must be determined *potentiometrically* (5). Thus the number of mols, n, of the HCl or the NaOH neutralized by the glycine or sulfanilic acid is given respectively by the expressions:

$$n = N - \frac{cH}{\alpha} \text{ for HCl; and } n = N - \frac{cOH}{\alpha} \text{ for NaOH}$$

The only assumption involved is that at the same normality the acid

TABLE II.

N HCl	E.M.F.	pH	cH	n
	mv.			
.320	285.	0.617	.2415	.041
.250	291.8	0.730	.1862	.0395
.200	298.5	0.842	.1439	.040
.150	307.5	0.992	.1019	.0390
.120	316.	1.133	.0736	.0405
.090	326.5	1.308	.0492	.0375
.060	344.5	1.608	.02466	.0345
.040	365.3	1.970	.01072	.0291
.025	388.	2.350	.00477	.0205
.018	400.5	2.558	.00277	.0152
.012	414.	2.800	.00159	.0104
.009	422.5	2.942	.00114	.00786
.006	434.	3.133	.000736	.00526
.003	455.5	3.492	.000322	.00268
.001	484.	3.98	.000105	.0009
.0000	598.	5.9	—	—
NaOH			cOH	
.004	766.	8.75	.0000056	.004
.009	790.	9.15	.000014	.009
.015	806.5	9.43	.000027	.015
.025	831.5	9.86	.000073	.0249
.0373	871.5	10.53	.00034	.0368
.040	895.	10.933	.00085	.0387
.060	964.	12.10	.01259	.040
.080	981.	12.383	.02415	.0397
.100	990.5	12.542	.03483	.0401
.130	1000.5	12.708	.05105	.0397
.180	1009.5	12.875	.0750	.0398
.250	1019.	13.033	.1079	.0405
.3511	1028.	13.183	.1524	.040

or alkali ionizes to the same extent in the presence of the glycine or sulfanilic acid as it does when these are absent.

Table I gives values of cH and cOH for various normalities of HCl and NaOH at room temperature, 20–22°C. By plotting N

TABLE III.

Sulfanilic acid.				Glycine.			
0.04 N		0.023 N		0.10 N		0.02 N	
pH	n	pH	n	pH	n	pH	n
				1.14	.097	0.913	.020
				1.483	.0864	1.033	.019
				2.26	.0544	1.183	.0194
				2.56	.0372	1.408	.0186
				2.86	.0236	1.638	.016
				3.033	.0178	2.01	.015
				3.233	.0142	2.192	.0115
				3.37	.0086	2.467	.0086
				3.55	.0057	2.622	.0066
				3.85	.00286	2.867	.00464
						3.20	.00237
						3.70	.0008
2.13	—	2.47	—	5.9	—	5.9	—
2.483	.004	3.033	.009			9.533	.00845
2.683	.009	3.50	.015			10.017	.0140
2.917	.015	3.54	.018			10.05	.0164
3.282	.025	11.18	.023			11.45	.0197
4.50	.040	12.03	.0226			12.07	.020
12.084	.0405	12.33	.0234			12.36	.0203
12.367	.0405						
12.553	.040						
12.70	.039						
12.88	.039						
13.033	.0395						
13.183	.04						

against cH or cOH, curves are obtained by means of which the normality of acid or base corresponding to any hydrogen or hydroxyl ion concentration may be read.

Titration of Glycine and of Sulfanilic Acid.—An N/10 solution of

glycine was prepared, and 10 cc. of this added to 15 cc. each of the various solutions of HCl and NaOH. The resulting glycine concentration was thus 0.04. Table II gives the results with glycine. N

TABLE IV.

0.04/0.043		0.02/0.02		0.10/0.04		0.04/0.08	
pH	n	pH	n	pH	n	pH	n
0.617	.041	1.033	.019	1.467	.0841	1.525	.0286
0.73	.0395	1.18	.0194	2.175	.0532	1.825	.0246
0.84	.04	1.41	.0170	2.433	.0363	2.117	.01724
0.99	.039	1.62	.015	2.625	.0226	2.208	.01174
1.125	.039	1.97	.014	2.73	.01614	2.333	.0092
1.31	.0375	2.15	.0108	2.833	.011	2.39	.0049
1.58	.033	2.32	.00715	2.88	.0077	2.48	.0027
1.91	.0273	2.405	.00506	2.97	.0049	2.55	.00017
2.21	.0186	2.525	.00301	3.017	.00204		
2.36	.0136	2.65	.00076				
2.48	.0087						
2.575	.00634						
2.625	.00363						
2.683	.00092						
2.74	—	2.76	—	3.06	—	2.60	—
2.8	.001	2.967	.004				
2.85	.003	3.217	.009				
2.933	.0075	3.583	.015				
3.083	.0133	8.867	.025				
3.483	.0266	10.38	.0397				
4.11	.039	12.067	.041				
4.517	.0412	12.367	.041				
6.14	.0433	12.55	.039				
8.25	.045	12.71	.0397				
8.70	.048	12.883	.039				
9.175	.054	13.03	.0405				
9.45	.060	13.183	.040				
10.15	.0748						
10.92	.082						
12.05	.0829						
12 32	0824						

is the original normality of the HCl or NaOH after dilution from 15 to 25 cc., and n is the number of mols neutralized by the glycine, per liter of mixture.

Table III gives, in condensed form, results obtained with certain concentrations of sulfanilic acid as well as certain other concentrations of glycine. The solubility of the sulfanilic acid prevented making an N/10 solution at room temperature, so the solution was made up at 40°C. using such a volume that 0.1 mol would occupy a volume of 1 liter at 20°C. A quantity was pipetted from this solution at 40° such that it would occupy a volume of 10 cc. at 20°. As may be expected, acid titration of sulfanilic acid has little meaning, since at the pH where any effect may be expected, a very small

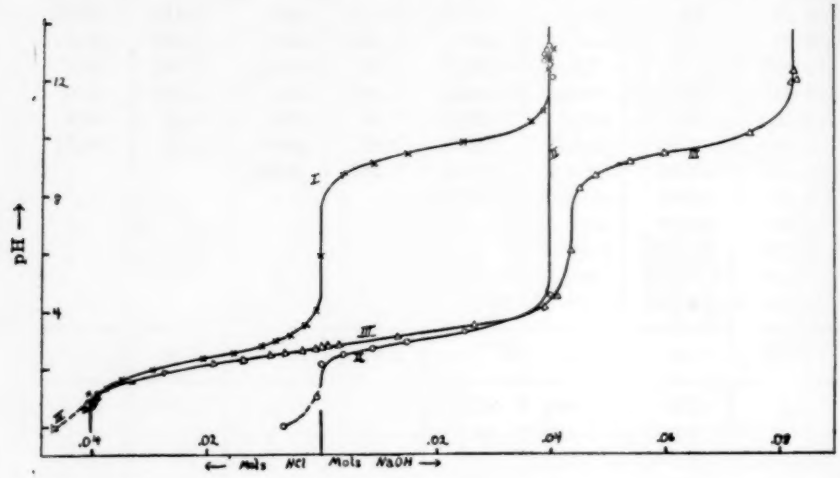

FIG. 1.

change in E.M.F. corresponds to a large quantity of sulfanilic acid neutralized. Thus, except for glycine, only alkaline titrations are included.

In Tables III and IV, n represents mols of HCl neutralized when it is above the center line, and mols of NaOH neutralized when below this line.

Table IV, which is analogous to Table III, gives results obtained from the titration of various mol ratios of glycine and sulfanilic acid. The concentration of glycine is given first in the ratios, *i.e.* the ratio 0.04/0.043 means a mixture containing 0.04 mol glycine and 0.043 mol sulfanilic acid per liter.

The curves in Fig. 1 are plotted from data obtained by titration of the 0.04 N glycine (Curve I), the 0.04 N sulfanilic acid (Curve II), and the 0.04/0.043 mixture of glycine and sulfanilic acid (Curve III). The alkaline titration of the sulfanilic acid (Curve II) is plotted on an abscissæ scale 43/40 that of the other curves to compare its behavior easily with that of the mixture. The broken portions of the curves are drawn, not through directly determined experimental points, but through points calculated from the basic ionization constant of sulfanilic acid (6). Abscissæ give the mols of HCl, measured to the right of the zero line, or of NaOH, measured to the left of this line, neutralized, as calculated by the formula given above, while ordinates give the corresponding pH. Similar curves may be obtained by plotting in a like manner the data from the other titrations.

The striking thing about the curve is the high buffering action of the mixture of glycine and sulfanilic acid about a pH which, it will be shown, corresponds closely with the value that one would calculate for what has been termed the isoelectric point of the system, from the formula developed in the first paper of this series. Moreover, it will be noted that the behavior of the mixture as a seeming individual, at least with a characteristic curve differing from the curves of the components studied alone, is confined to that portion of the pH range between the respective isoelectric points of the components. At a pH above the isoelectric point of glycine the curve representing the behavior of the mixture is an exact duplicate of the glycine curve, displaced to the right, of course, by the amount of the sulfanilic acid concentration in the mixture. In the same way there is no reason to doubt that the curve below the isoelectric point of the sulfanilic acid, at a pH of 1.25 (6), and that of the pure sulfanilic acid would also duplicate each other, though unfortunately, with this substance, that portion of the curve cannot be easily experimentally realized.

Calculation of the Amount of Glycine Neutralized by Sulfanilic Acid. —The total amount of glycine neutralized can be estimated by considering the expression

$$\overset{+}{H} + \text{glycine} = \overset{+}{Gl}\ \text{(glycine ion)} + HOH$$

where

$$\frac{\overset{+}{(Gl)}}{\underset{+}{(H)}\ (glycine)} = K$$

Reading the titration curve at various known ratios of glycine ion concentration to that of unneutralized glycine, K may be evaluated. For example, when the glycine is half neutralized, $K = 1/\overset{+}{(H)} = 223$, since 0.02 mol of glycine is seen to be neutralized at a pH of 2.35 or a cH of 0.0047. In a like manner for the following ratios the corresponding values of K are obtained.

Ratios.	pH	K
0.333	2.8	211.
0.500	2.64	218.
1.000	2.35	223.
2.000	2.05	224.
3.000	1.9	238.
Mean.....		223.

From the value of K the amount of glycine neutralized at any pH can be calculated. In the presence of NaOH this value is, through the pH range included in Table V, also the amount of glycine neutralized by the sulfanilic acid. When HCl is also present one must subtract from the total glycine neutralized the amount neutralized by the HCl. This can be satisfactorily approximated by putting it equal to the sum of the increase in glycine ion and the decrease in sulfonate ion at any pH, referred to their respective concentrations in the absence of the HCl.

Table V gives the results of such calculations for the mixture represented in Fig. 1. Above the center line the mixture contains NaOH, while below, it contains HCl. Glycine ion is represented by $\overset{+}{Gl}$, sulfonate ion by $\bar{S}$, and their respective tabulated increments by $\Delta\overset{+}{Gl}$ and $\Delta\bar{S}$.

The mols of glycine neutralized by the sulfanilic acid are seen to pass through a maximum when there is neither HCl nor NaOH present. Such a mixture has a pH of 2.74. The theoretical value for

the isoelectric point of such a system, it will be remembered from the first paper of this series, is obtained from the expression

$$(H)^+ = \sqrt{\frac{K_a\,(HAOH)}{K_b'\,(HBOH)}\,K_w}$$

where, in the present case, K_a is the acid ionization constant of the sulfanilic acid, 7×10^{-4}, and (HAOH) is its concentration in unionized

TABLE V.

Concentration of HCl or NaOH.	cH	$\dot{G}l$	$\bar{S}$	$\Delta\dot{G}l$	$\Delta\bar{S}$	Glycine neutralized by HCl.	Glycine neutralized by sulfanilic acid.
.0267	.00033	.00273	—	—	—	—	.00273
.0133	.00083	.0062	—	—	—	—	.0062
.0075	.00117	.00825	—	—	—	—	.0083
.0030	.00141	.00954	—	—	—	—	.0095
.00133	.00158	.01040	—	—	—	—	.01040
.0000	.00183	.01155	.0119	—	—	—	.01155
.001	.00186	.0117	.01175	.00015	.00015	.00030	.01140
.002	.00191	.0119	.0115	.00035	.0004	.00075	.01115
.003	.00207	.0126	.01085	.00105	.00105	.0021	.0105
.006	.00237	.0138	.0098	.00225	.0021	.00435	.00945
.009	.00266	.0149	.00895	.00335	.00295	.0063	.0086
.012	.00329	.0169	.00755	.00535	.00435	.0097	.0072
.018	.00439	.0197	.0059	.00815	.0060	.01415	.00555
.025	.0062	.0232	.00436	.01165	.00754	.01919	.0040
.040	.0124	.0294	.0023	.01785	.0096	.02745	.00195
.060	.0261	.0341	.0011	.02255	.0108	.03335	.00075

form; and K_b' is the basic ionization constant of the glycine, 2.2×10^{-12}, and (HBOH) is its concentration in unionized form. Substituting the proper values, one is lead to the theoretical pH value for the isoelectric point of this mixture of 2.73 in place of the observed value of 2.74.

Other mol ratios yield analogous results. Table VI compares the observed pH of the isoelectric points of various mixtures of glycine and sulfanilic acid with the calculated values. These calculated theoretical values, it will be remembered, correspond to the pH at which the negative sulfonate ion concentration is equal to the posi-

tive glycine ion concentration. They are obtained by solving the following two simultaneous equations for y.

$$\frac{y \cdot x}{c - x} = K_a \quad \text{and} \quad \frac{K_w/y \cdot x}{c' - x} = K_b'$$

where y is the hydrogen ion concentration corresponding to the pH of the isoelectric point of the system;

x is the sulfonate (or glycine) ion concentration at this pH, which is the same in case of both ions;

c is the total sulfanilic acid concentration, and K_a its acid ionization constant;

c' is the total glycine concentration, and K_b' its basic ionization constant.

TABLE VI.

Concentration of sulfanilic acid.	Concentration of glycine.	pH observed.	pH calculated.
.043	.040	2.74	2.73
.01	.09	3.40	3.44
.03	.07	2.98	3.01
.04	.10	3.06	3.03
.05	.10	2.98	2.97
.10	.10	2.74	2.75
.02	.02	2.76	2.75
.10	.05	2.58	2.54
.08	.04	2.57	2.54
.07	.03	2.51	2.50

Table VII gives certain results of the same nature as those included in Table VI but for a few other pairs of substances. In connection with the work presented in the following paper a sample of lysine was prepared from hydrolyzed casein. The ionization constants of this substance are evidently not very accurately known, probably since it is difficult to prepare it with a high degree of purity. Scudder (7) gives for K_a about 1×10^{-11}, and for K_b "less than 1×10^{-7}." From Tague's titration of lysine dihydrochloride (8) one may calculate the value of K_a, which is found to be 1.2×10^{-11}, but, though the second basic ionization constant can be obtained from his curve, the first, which is the significant one, cannot. Solutions of the sample prepared for this work gave a pH to water of 8.8, which, using

1.2×10^{-11} for K_a, gives the value 5×10^{-8} for the first K_b. It is almost impossible to be sure that one has not a trace of sulfuric acid in such a preparation, however, and for the calculations of the theoretical pH values given in Table VII the value 7×10^{-8} was used. The agreement between the observed and calculated values of the isoelectric points of these systems, though still quite satisfactory, is not so good as in Table VI. This may be due partly to the problematical value of K_b, for lysine used in calculating the theoretical values, and partly to the fact that small changes in the mol ratio have a much larger effect on the pH than is true for glycine and sulfanilic acid, and thus small errors in the total concentration, which may be the case for the lysine, will contribute largely to the discrepancies. The agreement is, however, considered good.

TABLE VII.

	pH observed.	pH calculated.
0.04 sulfanilic : 0.02 lysin.....	3.09	3.15
0.02 " : 0.02 "	4.88	5.0
0.02 " : 0.04 "	6.69	6.82
0.02 glycine : 0.01 lysin.....	8.05	8.09

It will be noted that in the last instance the glycine is playing the rôle of the acid constituent of the mixture, as it is now at a pH above its isoelectric point.

SUMMARY.

Electrometric titrations of glycine, sulfanilic acid, and various mixtures of the two have been made. These mixtures are shown to give a curve which, between their respective isoelectric points, is different from that of either substance. These mixtures have a maximum buffering power at a pH which can be theoretically calculated, and which has the characteristics of an "isoelectric point of the system."

Other pairs of ampholytes are shown to act in an analogous manner.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

XII. The Metabolism as a Function of Age.

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The rates of absorption, storage, and elimination of energy are perhaps the best indices that we possess of the vitality of a living organism. Since it has been frequently verified that: [*A*] absorbed energy = [*S*] stored energy + [*E*] eliminated energy; and since, by the chemical analyses of embryos at successive ages, the rate of storage has been ascertained (1), it remains only to obtain the catabolic activity of the embryo in terms of age. This has been done by the manometer method for the estimation of oxygen, and the present communication reports the results. The initial estimations of CO_2 production reported elsewhere (2) were admittedly subject to a number of unknown variables determinable with difficulty, such as variations in the concentration of CO_2 in the embryo and in the albumin and yolk, together with the contribution made by the carbonates dissolved from the shell. These values are functional to the carbon dioxide tension about the egg, and since, moreover, they cannot be estimated with great precision, a statistical analysis of the data from a very large number of eggs would be necessary. For these reasons we chose to measure metabolism by the oxygen consumption.

Method.

A Warburg manometer was used, attached to a special glass vessel to contain the hen's egg. The egg rested upon glass tips projecting from the walls to suspend it above the bottom which was layered with 5 cc. of a 1.0 N NaOH solution.

The volume of each apparatus, and thus its constant, was obtained by the method of Warburg (3). Brodie's fluid was used in the manometer. The volume occupied by the egg contents in the vessel, *i.e.* the whole egg minus the air sac, was assumed to be equal to the weight of the egg minus the weight of the shell (approximately 6 gm.). In general, eggs of the same size and shape were selected.

A control was made with each test; at first in the form of a fertile egg of 1 day incubation, but later, when this was found unnecessary, with a vessel empty except for the alkali. The experiments were done in a constant temperature room, eliminating thereby the use of a water bath.

A thermometer ground into each glass cover registered the temperature, which averaged approximately 39.0°C. The small fluctuations which did occur were not found to affect appreciably the results. Moreover, it was found that the large surface of alkali exposed provided for maximum absorption of CO_2 without the necessity of shaking. Shaking for a minute prior to reading the manometer made no difference in the result. Nor was it found, when thin rubber tubes in which cold water flowed were run along one side of the vessel to cool the wall over this area and thereby to initiate by convection a regular circulation, as in Barach's (4) recently constructed human oxygen chamber, that any acceleration of CO_2 absorption took place.

About $\frac{1}{2}$ hour (the length of time being judged by the behavior of the control) was allowed for conditions to reach equilibrium. After this time readings were taken at varying intervals during periods of 2 to 6 hours until repetition of approximately similar results made one confident of their reliability. During the intervals between tests, the manometer and vessel were connected with an oxygen bag, so that the concentration of oxygen within the vessel remained always the same.

To obtain values for the rate of oxygen absorption per gm. of body weight the following figures are necessary, (1) the constant for the vessel (previously calculated); (2) the manometer readings, (3) the weight of the whole egg, and (4) the weight of the embryo.

One phenomenon was observed which we have not been able to explain. The embryo of an incubation age over 16 days, even when connected with the oxygen bag, did not survive in the apparatus over 12

hours. Their metabolism after 3 to 6 hours gradually fell. If the vessel was fully opened to the air for a few minutes the embryo would revive. Apparently it had nothing to do with a lack of oxygen, accumulation of CO_2, or changes in the humidity; neither was there an accumulation of ammonia.

TABLE I.

Metabolism of Chicken Embryos as a Function of Age.

1 Age.	2 No. of observations.	3 By experiment. O_2 per day per gm. wet weight.	4 Standard deviation.*	5 From curve. O_2 per day per gm. wet weight.	6 O_2 per day per gm. dry weight.	7 Solid oxidized per day per gm. dry weight.	8 Solid stored per day per gm. dry weight.	9 Absorption per day per gm. dry weight.	10 CO_2 per day per gm. wet weight.	11 Resp. quot.
days		*cc.*		*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*cc.*	
6	8	50.0	2.1	50.0	896	0.444	0.665	1.11	29.9	0.60
7	5	38.4	2.3	43.0	735	0.364	0.584	0.95	29.6	0.69
8	3	36.7	1.5	39.0	628	0.311	0.510	0.82	29.3	0.75
9	10	39.2	1.3	36.5	562	0.278	0.478	0.75	29.0	0.79
10	7	32.8	1.2	35.0	500	0.248	0.465	0.71	28.5	0.81
11	7	33.9	1.2	34.0	442	0.219	0.465	0.68	28.0	0.82
12	9	33.9	1.2	33.2	378	0.187	0.465	0.65	27.0	0.81
13	5	32.4	0.7	32.5	322	0.159	0.465	0.62	25.7	0.79
14	5	30.3	0.6	31.7	259	0.128	0.447	0.57	24.0	0.76
15	7	34.3	1.3	30.5	209	0.103	0.395	0.50	22.0	0.72
16	11	29.0	1.4	28.7	175	0.087	0.320	0.41	20.1	0.70
17	5	26.1	1.0	26.2	152	0.075	0.250	0.33	18.1	0.69
18	6	21.1	1.3	23.2	131	0.065	0.215	0.28	16.2	0.70
19	3	20.5	0.3	20.0	113	0.056			14.2	0.71

Column 6 = figures calculated by the aid of values for the percentage of solid substance previously determined (Murray (1)).

Column 7 = values in Column 2 divided by 2019.3 (amount of oxygen absorbed when 1 gm. of fat is burned).

Column 8 = figures previously obtained (Murray (1), Table III).

Column 10 = figures read from smooth curve previously obtained (Murray (5)).

RESULTS.

The results of the oxygen determinations (Table I) may be seen (Fig. 1) to demonstrate a decrease in metabolic rate per gm. of body weight with age. This conclusion confirms that reached when the carbon dioxide was determined; except that in a more precise analysis

and comparison of the results it appears that the oxygen estimations show a sharp fall of metabolism during the first days of the period under observation, whereas the carbon dioxide figures do not (5). As has been mentioned in the introductory remarks, however, numerous complications arising from the variability of unknown factors cast doubt upon the value of CO_2 elimination as a measure of catabolic change.

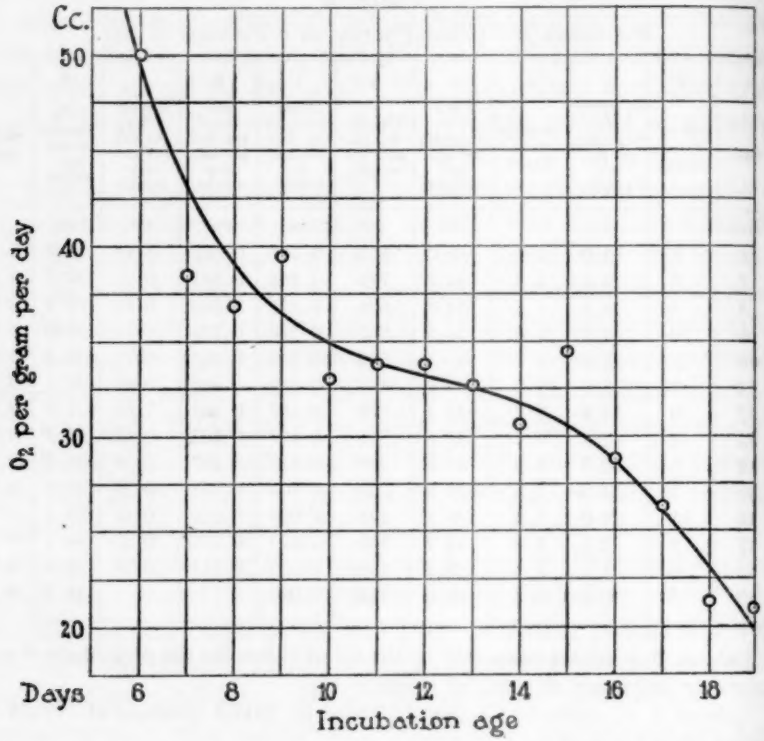

FIG. 1. Oxygen consumption in cc. per gm. of wet weight of chick embryo per day, as a function of age.

The oxygen determinations on the other hand were well controlled and presented no obvious factor to vitiate their use as indices of metabolic activity.

To oxidize 1 gm. of fat approximately 2000 cc. of O_2 are absorbed; whereas to oxidize 1 gm. of protein (966.3 cc.) or starch (828.8 cc.) about 900 cc. of O_2 are used (6). The total oxygen consumption for

the first 19 days, estimated by graphical integration, comes to 2988 cc., which on the basis that only fat is burned during incubation leads to the conclusion that 1.48 gm. of dry substance (*i.e.* fat) is oxidized during that period. If only protein and starch were burned, it would require over 3.28 gm. to use the observed amount of oxygen. Previous chemical analyses have shown that approximately 1.62 gm. of substance is burned during the first 19 days, a figure which may now be accounted for on the assumption that 92 per cent of the metabolism is oxidation of fat, and the rest of protein and carbohydrate. This value is to be compared to 98 per cent fat oxidation found by measuring the CO_2 output. The former figure is probably more accurate.

During the last 5 days of incubation, when about four-fifths of the total oxidation takes place, the respiratory quotient is approximately 0.71, which points to fat consumption, during this period. The earlier values for the respiratory quotient are somewhat higher (up to 0.81); but they are variable and it is uncertain whether they deserve consideration. The results point to some error during the first 3 days when the CO_2 figures, and thus the quotient, also seem to be definitely too low.

If we discard the carbon dioxide estimations in favor of these later O_2 determinations and assume as we may without undue error that catabolism is at the expense of fat, we arrive at some notion of the changes in the metabolic rate with age.

Regarding the organism energetically and dynamically, the amount of energy exchange measures its activity or vitality. Hence, the amount of energy stored plus the amount set free might be used as a criterion of aliveness. By adding the rate of storage in terms of weight (previously obtained) to the rate of elimination, likewise in terms of weight as measured by oxygen usage, one obtains the desired value; namely, the rate of dry mass absorption per gm. of body weight per day (Table I). It may be seen (Fig. 2) that there is a marked fall with age in the rate of absorption expressed in these terms. Reasons have been enumerated for believing that during the first half of incubation, when the amount of metabolism is small relative to the total metabolism during incubation but large relative to the weight of the embryo, there is a not inappreciable amount of protein and carbohydrate oxidized. If this were a fact a straight line rather than an S-shaped curve as graphically represented might be indicated.

The consumption of food during the early days is enormous. On the sixth day for instance the embryo absorbs over its own mass of dry substance. Assuming that the water content of the diet is ap-

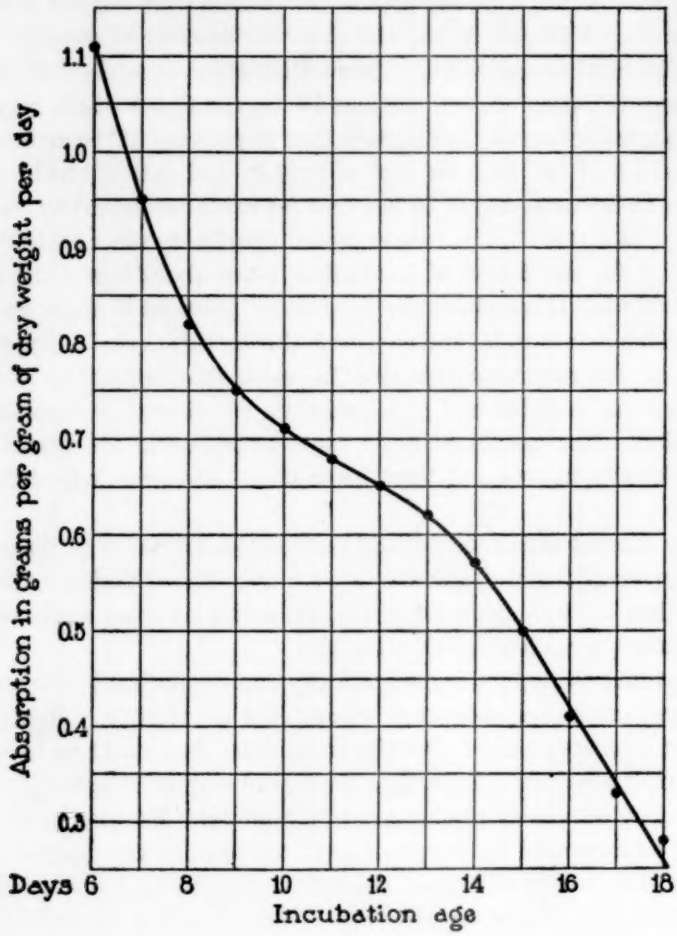

FIG. 2. Absorption of solid matter in gm. per gm. of dry weight of chick embryo per day, as a function of age.

proximately that of the tissues, this would be equivalent to a mature man eating about 150 pounds of food per day. During the 12 days under observation, however, the percentage rate of absorption falls

to about 25 per cent (one-fourth its earlier value). According to Lotka, a mature meadowlark consumes about 6.6 per cent of its own weight a day (7) which would suggest a fall in absorption rate during the postembryonic period of a degree comparable to that which occurs during the 12 days before hatching.

SUMMARY.

1. The previous findings that the rate of metabolism per gm. of body weight decreased with age, and that during the incubation period catabolism was mostly at the expense of fat, have been confirmed.

2. These determinations of the rate of oxygen uptake have afforded more precise values for the catabolic rate and thus permit estimations of the changes with age in the rate of absorption.

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REGULATION OF THE HYDROGEN ION CONCENTRATION AND ITS RELATION TO METABOLISM AND RESPIRATION IN THE STARFISH.

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The composition and condition of the body fluids of animals show departures from those of their environment usually corresponding to their degree of development. In the cœlenterates and sponges a free circulation of sea water accomplishes the distribution of materials. The interior and exterior are then exposed to practically the same medium. In the starfish, however, there are a closed cœlomic cavity and a digestive system which are not swept by a constantly renewed supply of sea water. The fluids contained in these spaces are similar to sea water, but modified by the enclosing tissue so as to be true components of the organism. The hydrogen ion concentration of the body fluids becomes progressively greater in the deeper regions of the organism, establishing a gradient from the interior toward the exterior. The normal reactions of these fluids are shown here to be optimal for several representative metabolic processes.

In the starfish there is a mouth-opening situated on the lower side, through which the soft cardiac portion of the stomach is extruded to surround food. In this stomach digestion proceeds until the food is sufficiently disintegrated to permit its withdrawal into the body. The partially digested food is then swept by ciliary currents through the pyloric cæca, where digestion and absorption are effected. Then, apparently, the products of digestion pass through the wall of the cæcum into the surrounding cœlomic fluid. Here again ciliary currents, together with body movements, serve for distribution (Irving, 1924–25).

The investigations presented were carried out with two forms of

starfish common in the vicinity of the Hopkins Marine Station at Pacific Grove, California. These forms were *Pisaster ochraceus* and *Patiria miniata*. *Patiria* is short-armed, thin-walled, and much more tractable in aquaria. It was therefore used for most of the experiments, although many of them were also tried on *Pisaster* with corresponding results.

Normal pH of Stomach, Cæca, and Cœlomic Fluid.

The following preliminary experiments showed that the stomach is distinctly more acid than sea water. Mussel meat stained with neutral red takes the deep red color of a pH less than 7. During cardiac digestion of such meat, the red color is retained, and the stomach walls take and keep for several days a pink color indicating a hydrogen ion concentration near neutrality. Several times I was able to pipette enough fluid from the stomach through the mouth to show a pH between 7.3 and 7.5 with phenol red. This fluid is already much more acid than the sea water with which it is in such relatively close contact.

After ingestion of stained mussel meat the oral lumina of the cæca stain deep red with neutral red, the remaining regions being naturally too dark to show the dye. The red color remains conspicuous in the lumen for a week, during which time this particular cæcum region plainly remains more acid than sea water, but not far from neutrality. Although the neutral red color in the dark cæca is difficult to compare directly, the stained cæcum changes color distinctly when placed in a more alkaline or acid buffer solution. A series of cæca stained by ingestion of neutral red were placed in buffer solutions, and, from observation of the solution producing the least change, the cæcum pH was estimated to lie between 6.6 and 7.0. This is quite contrary to the observations of Roaf (1909–10[1]) who declares that the cæca of *Asterias rubens* become alkaline after digestion is completed.

Samples of cœlomic fluid, which surrounds the cæca in the body cavity, showed the following acidities:

Patiria	pH	8.0	7.7	7.6	7.6	7.6	7.6
Pisaster	pH	7.5	7.6	7.5	8.1	7.6	7.6
Sea water	pH	8.3					

[1] Roaf (1909–10), p. 448.

These are typical of many determinations taken at various periods before and after feeding, and, considering the difficulties from mixture with sea water and a slight opalescence, indicate a consistent normal close to pH 7.6. McClendon (1916–17) found the pH of cœlomic fluid of the sea urchin *Toxopneustes variegatus* between 7.7 and 7.8, while Crozier (1918) found the cœlomic fluid of the holothurian

TABLE I.

Changes in the Hydrogen Ion Concentration of Sea Water Caused by Excised Cæca.

Experiment No.	0 hrs.		20 hrs. Cilia condition.		45 hrs. Cilia condition.	Comparative activity.
	pH	*pH*		*pH*		
1	5.0	6.0	Dead.	5.8		0
2	5.6	6.3	o.s. active. i.s. dead.	5.8	o.s. slightly active. Digestion started.	1
3	6.0	6.35	o.s. active. i.s. active.	6.0	o.s. slightly active. i.s. dead.	2
4	6.2	6.35		6.2	o.s. active. i.s. dead.	3
5	6.4	6.4		6.2	o.s. active. i.s. slightly active.	4
6	6.6	6.35		6.2	o.s. active. i.s. slightly active.	3.5
7	6.8	6.55	o.s. active. i.s. active.	6.2	Dead.	0
8	7.0	6.85		6.7	o.s. slightly active. i.s. dead.	1
9	7.4	6.95	o.s. active. i.s. active.	6.8	o.s. active. i.s. active.	4
10	7.8	6.9		6.7	o.s. active. i.s. dead.	1
11	8.4	6.9	o.s. active. i.s. active.	6.4	o.s. active. i.s. dead.	1

o.s. = outside, toward cœlom; i.s. = inside.

Stichopus mœbii at 7.6. Cœlomic fluid is also different from sea water in its salts. The freezing point depression of a sample from *Patiria* was determined by Dr. J. P. Baumberger to be 1.885, compared with 2.075 for sea water.

These observations show that the fluids and organs of *Patiria* and *Pisaster* are maintained at an acidity quite different from that of sea water.

The Optimum for Survival.

After the discovery that the internal organs and fluids have each a normal, regular pH, they were next subjected to environments varying in this respect. In order to start under reproducible conditions, sea water acidified with HCl was aerated until a constant pH was reached, indicating the arrival of the solution at carbon dioxide equilibrium with the air.

TABLE II.

Changes in the Hydrogen Ion Concentration of Sea Water Produced by Excised Cæca.

Experiment No.	Start.	17 hrs.	21 hrs.	45 hrs.
	pH	*pH*	*pH*	*pH*
1	5.0	6.2	6.1	6.0
2	5.4	6.3	6.3	6.1
3	5.8	6.3	6.3	6.1
4	6.0	6.7	6.7	6.2
5	6.2	6.3	6.3	6.1
6	6.4	6.3	6.3	6.2
7	6.6	6.3	6.3	6.1
8	6.8	6.7	6.7	6.7
9	7.0	6.8	6.7	
10	7.2	6.8	6.7	
11	7.4	6.8	6.7	6.6
12	7.6	6.8	6.8	6.6
13	7.8	6.8	6.8	
14	8.0	6.6	6.5	
15	8.2	6.7	6.7	
16	8.4	6.7	6.7	

To such solutions in test-tubes, excised cæca were added, one in each tube. Table I shows the changes which characteristically occurred in these experiments, and Table II extends the results. The condition of the tissues was determined in each case by examination with a binocular dissecting microscope.

These experiments show a tendency of the excised, living cæca to alter the medium toward a pH of about 6.7. When the pH fell below 6.7 to about 6.3, disintegration and injury to the cilia were apparent. The halt at pH 6.7 is too distinct to represent gradual

decomposition, and the subsequent rapid fall to pH about 6.3 indicates a change to a new status. Probably the lower pH, which is concomitant in appearance with physical signs of tissue death, represents the stage of autolysis. It is apparent that the living animal maintains a rather definite normal reaction of its cæca, and that the cæca themselves when excised are capable of producing the normal reaction in sea water media initially quite different.

All of these tests dealt with living tissue, except as indicated. It was not a matter of autolysis or putrefaction because of the persistence of ciliary action and the absence of the conspicuous odor of dead marine animals. These soft tissues disintegrate and produce unpleasant odors almost immediately after ciliary action ceases.

The Optimum pH for Digestion.

Many of the older physiologists investigated the problem of digestion in echinoderms and produced conclusions of quite contradictory nature. Abderhalden (1911[2]) lists seven different enzymes named in echinoderms by various workers. The names appear, however, to be usually assigned by analogy with mammalian enzymes on the basis of substance digested and conditions of action, quite apart from normal conditions. Beyond these analogies, based often on rather indefinitely reported observations, there is little precise evidence of the nature of the enzymes. It is clear that the cæca produce digestive enzymes (Frédericq, 1878; Cohnheim, 1901; van der Heyde, 1922, 1923). These enzymes easily bring about autolysis of the organs, but extracts behave in an extremely slow fashion. Furthermore, the technique applied to their study has not been of the sort calculated to give quantitative results.

Frédericq (1878) first clearly showed that proteolytic digestion by the cæca occurred near the neutral reaction. Other invertebrates probably carry on digestion naturally near neutrality, and not in the strongly acid condition where pepsin finds its optimum. Darwin's observations on digestion in the earthworm show a normal reaction near neutrality (Darwin, 1890). Bodansky and Rose (1922[3]) extracted

[2] Abderhalden (1911), p. 538.
[3] Bodansky and Rose (1922), p. 475.

a digesting substance from jelly fish which had one optimum for gelatin liquefaction at pH 2.6, and another, much more favorable, at pH 7.3. The optimum at pH 2.6 seems too far removed from the normal reaction of sea water, which circulates so freely through the animal, to be important under vital conditions. The empty stomach of *Stichopus* was found by Crozier (1918) to have a pH between 5.0 and 6.5, while during feeding it ranged from 4.8 to 5.5. Crozier remarks that this animal's calcareous diet may constitute a special condition which is met by the relatively strongly acid digestive juices. Roaf's observations of cH 10^{-4} during digestion in *Asterias rubens*[1] suggest an acidity far greater than any which I observed in *Pisaster* or *Patiria*, although they are similar forms. Van der Heyde (1922) observed in the stomach of *Asterias* pH 7.1, 7.6, and 7.7, and in the cæca 7.3. The chemical methods and biological conditions involved in determining the normal hydrogen ion concentration during digestion must be rather precisely established, so that many casual observations require critical examination. From the available evidence and the biological conditions a natural reaction far from neutrality would be unexpected.

Because of the difficulties found in separating active extracts, digestions by excised surviving cæca *in vitro* were used. These are here nicely applicable because of the appropriateness of the cæca and the excellent criterion of their survival in ciliary activity.

Pipettes were prepared with their tips protected by rubber tubing, the opposite end bearing a short length of rubber tubing and a pinch-cock. 2 cc. of the solution to be digested were introduced into the pipette, its lower end stopped, and the pinch-cock closed. By slightly withdrawing the closed rubber tube from the pipette the solution could be retained without loss. Next the rubber-protected end of the pipette was inserted into the open (pyloric) end of a previously weighed cæcum and the cæcum tied on with silk. The cæcum was then drained and transferred to a test-tube containing 10 cc. of sea water prepared at constant pH. The pipette and cæcum were suspended in the solution by a cork, the pinch-cock opened, and the solution allowed to run into the cæcum. The cæcum became distended and remained distended, if uninjured, for 2 days or more. In this way a sack of living digestive tissue was produced, with a

capacity in the case of large cæca of over 3 cc. Products of protein digestion diffused through the cæcum wall and were determined by the Van Slyke method for amino nitrogen.

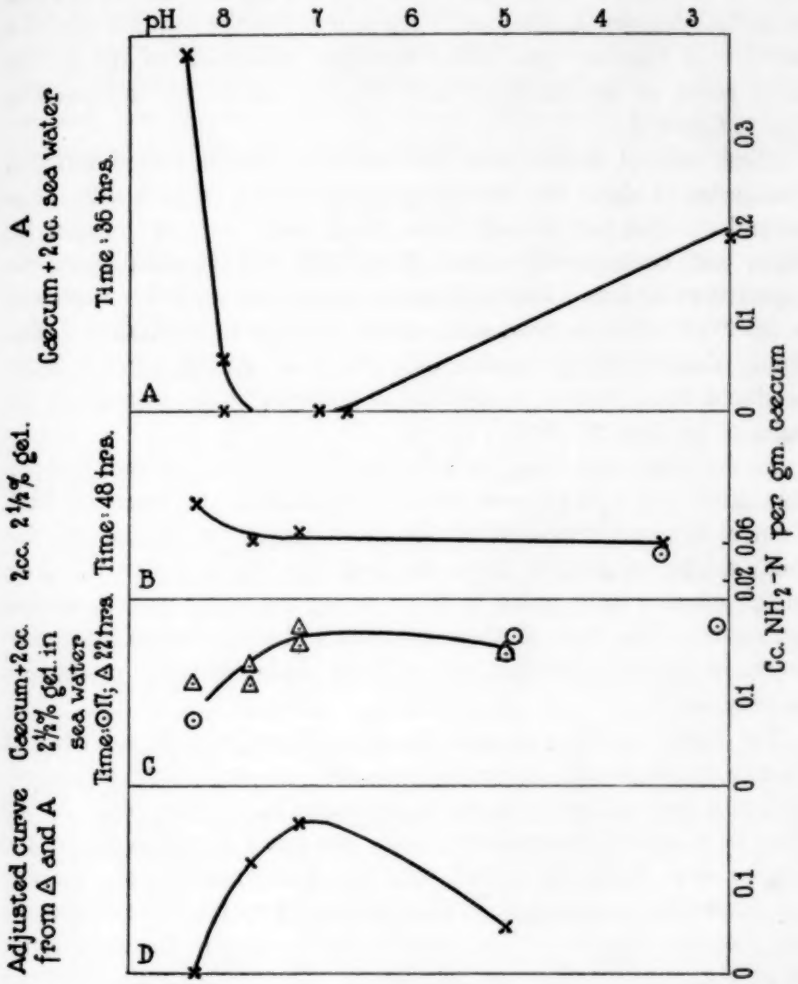

FIG. 1.

An appreciable production of amino nitrogen might be expected from the cæca themselves. In order to determine the normal amino

nitrogen production, 2 cc. of sea water acidified and equilibrated by aeration were introduced through the pipette into the cæcum. The cæcum was then immersed in 10 cc. of the same solution. After an interval of time a 2 cc. sample of the external liquid was removed and its amino nitrogen determined. The new pH of the solution was also noted. In this way the amino nitrogen production of the cæcum alone could be determined. Data for the results are indicated in Fig. 1, Curve A.

These control experiments indicate a minimum amino nitrogen production at about the neutral point, increasing on each side. The neutral reaction has already been shown to be near the normal for cæca, and consequently tissue death and self-digestion would be expected to be less. Bacterial action is not excluded, but if present is least effective in producing amino nitrogen at neutrality. Evidently also the energy consumed by the tissue during survival is not produced from protein, or the amino nitrogen would increase at the point of greatest activity.

To determine the effect on food digestion of various hydrogen ion concentrations, a $2\frac{1}{2}$ per cent solution of gelatin in sea water was used. Control determinations of the amino nitrogen production of this stock solution during 48 hours are shown in Fig. 1, Curve B. 2 cc. of the solution were added to 10 cc. of the sea water used in the experiments. The time of these controls is double that in the experiments on gelatin digestion, and yet the amino nitrogen production is very small.

The digestive effect of cæca on gelatin was tried in solutions at various hydrogen ion concentrations. All sea water used was acidified with hydrochloric acid and then aerated to constant pH. At the time of digestion determination, pH was again determined and the results were found in accord with previous experiments. Results are indicated by Curve C in Fig. 1, where two typical experiments are recorded together. The curve presents directly the appearance of an optimum for digestion near pH 7. The greater amino nitrogen production in acid reactions is obviously, from Curve A, attributable to autolysis. When a curve is adjusted by deduction of amino nitrogen production as in Curve A from that in Curve C, the resulting curve, D, plainly shows an optimum near pH 7.

After these indications that the optimum digestion conditions were also those of the normal organism, attempts were made to prepare a digestive extract whose activity might be compared at different acidities. Various extracts were prepared by permitting cæca to autolyze with alcohol or toluol. Products of such procedure caused only slow digestion, and the results did not show that any appreciable separation of the enzyme had been effected. As another method, many cæca were macerated with sand, spread on glass plates, and dried under low pressure at 40 degrees. The dried mass

TABLE III.

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals.

	Experiment No.	Weight of cæca.	Start.	Period 1.		Period 2.		Period 3.	
		gm.	*pH*	*hrs.*	*pH*	*hrs.*	*pH*	*hrs.*	*pH*
Aerated.	1	3.64	8.2	3.08	7.8	9.40	7.7	3.16	7.8
	2	4.37	8.0	3.00	7.5	9.40	7.4	3.16	7.3
	3	3.53	7.8	2.92	7.4	9.40	7.3	3.16	7.3
	4	5.34	7.3	2.84	7.1	9.40	7.3	3.16	7.2
	5	4.53	5.0	2.80	6.8	9.40	6.8	3.16	6.8
	6	4.53	3.8	2.70	6.1	9.40	6.4	3.16	6.5
Sealed.	7	6.33	8.1	2.92	7.9	3.25	7.5	20.0	6.8
	8	6.58	7.9	2.75	7.7	3.25	7.3	20.0	6.7
	9	6.13	7.7	2.66	7.4	3.25	7.1	20.0	6.7
	10	6.41	7.3	2.56	7.1	3.25	6.8	20.0	6.6
	11	6.42	4.9	2.25	6.2	3.25	6.5	20.0	6.4
	12	6.25	3.8	2.16	4.8	3.25	5.2	20.0	5.6

was then ground, but could not be redissolved or uniformly suspended on account of the fat present. Using this material in temporary suspension, the neutral reaction was obviously most favorable for the liquefaction of gelatin.

The Optimum pH for CO_2 Production.

Having shown that the optimum reaction for ciliary survival and digestion corresponds with the normal tissue reaction of about pH 6.7, it would be expected that this reaction would be most satisfactory for metabolism in general. Respiration, measured by CO_2 production, is the indicator used here for metabolic activity.

For the experiments uniform amounts of weighed cæca were placed in Pyrex flasks with sea water of known pH in equilibrium with the air. One series was aerated, the other sealed with rubber stoppers to retain CO_2 as produced by the cæca. Changes in the sea water of the aerated series should be then attributed to non-volatile substances or to a buffer effect of the tissues themselves; changes in the sealed series would include these and the change from CO_2. During the experiments none of the tissues putrified, and the times were all less than had been previously shown quite compatible with survival.

TABLE IV.

Changes in pH of Sea Water Produced by Cæca during Different Time Intervals.

	Experiment No.	Weight of cæcum.	Start.	Period 1.		Period 2.		Period 3.		Period 4.	
		gm.	pH	hrs.	pH	hrs.	pH	hrs.	pH	hrs.	pH
Sealed.	1	1.80	8.6	5.59	7.7	1.75	7.6	2.25	7.6	12.9	7.1
	2	1.46	8.5	5.40	8.0	1.75	7.8	2.40	7.6	12.9	7.1
	3	1.62	8.5	5.25	7.6	1.75	7.3	2.40	7.1	13.0	6.8
	4	1.34	8.2	5.08	7.1	1.59	6.8	2.40	6.7	13.0	6.7
	5	1.38	5.0	5.00	6.4	1.50	6.5	2.40	6.5	13.0	6.4
	6	1.28	4.8	4.75	5.0	1.50	5.8	2.40	6.1	13.0	6.2
Aerated.	7	1.88	8.6	5.92	8.3	1.42	8.3	2.75	8.3	12.4	7.8
	8	1.82	8.5	5.75	8.3	1.42	8.3	2.75	8.3	12.6	8.2
	9	1.61	8.5	5.59	8.2	1.42	8.2	2.75	8.1	12.6	7.8
	10	1.36	8.2	5.25	7.7	1.42	7.7	2.75	7.3	12.6	7.2
	11	1.38	5.0	5.00	7.2	1.42	7.3	2.75	7.0	12.6	6.8
	12	1.37	4.8	4.84	6.3	1.42	6.3	2.75	6.2	12.6	6.4

The results of these experiments, recorded in Tables III and IV, show in both the sealed and aerated series the previously observed progressive acidification of the solutions above pH 6.7 toward this reaction. In the aerated series the change is relatively slight after the first period, an effect which probably originates principally in neutralization by tissue substance.

In preparing acidified sea water solutions the excess base, and consequently buffer effect, were changed by addition of strong acid. Therefore only those experiments are comparable which were carried out with the same solution. These are recorded in the tables in the

same horizontal line. In such solutions of similar excess base, hydrogen ion concentration change is directly proportional to carbon dioxide change in the range considered (McClendon, Gault, and Mul-

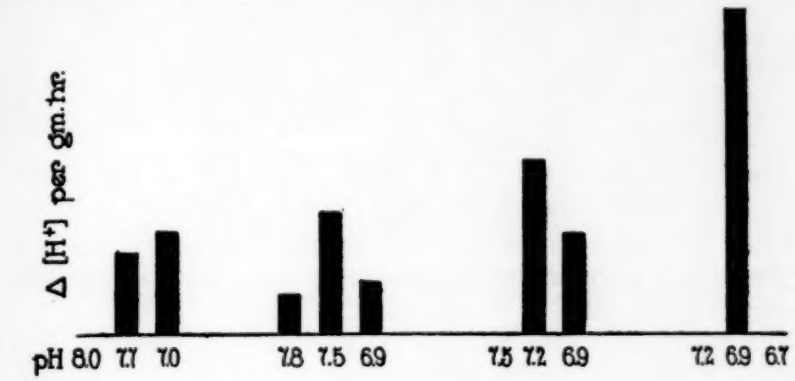

FIG. 2 (from Table III). Δ[H+] per gm. per hour in sealed series, corrected by subtraction of Δ[H+] of aerated series.

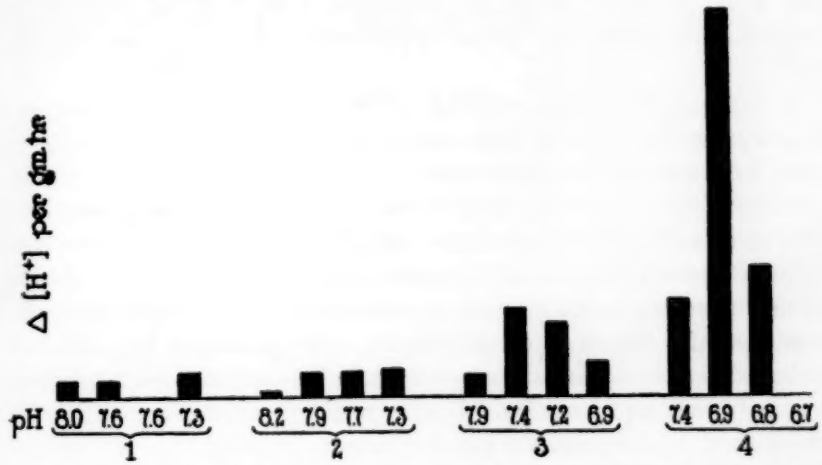

FIG. 3 (from Table IV). Δ[H+] per gm. per hour in sealed series, corrected by subtraction of Δ[H+] of aerated series.

holland, 1917). The progress of these changes in the sealed series is indicated in Figs. 2 and 3, where vertical height represents change in hydrogen ion concentration per gm. of cæcum substance per hour.

These are corrected by subtraction of the change in the corresponding aerated experiment.

The figures show in each experiment a small carbon dioxide production in the first period, followed by a much greater production in the second period, and usually by diminishing changes in subsequent periods. That respiration during the first period was so small is remarkable. It is apparent that the cæcum fits its medium better after some time for adjustment.

The reaction about pH 7, close above that found normal for the cæca, seems most favorable to carbon dioxide production. It is not possible, however, to judge this point; for the diminished excess base of the initially more strongly acidified series causes a correspondingly decreased buffer value.

The experiments show that carbon dioxide is the principal acid serving to bring the cæca to the normal reaction. The aerated series also changes slightly toward the normal reaction, but this change occurred almost entirely in the first period and may be attributed to the buffer effect of the cæcum substance. No evidence appears to suggest the production of an appreciable amount of non-volatile acid.

Conditions of acidity represented by pH less than 6.7 have already been shown to be unfavorable to the cæca. Where the reaction fell below pH 6.7 a sharp drop was noticed to a rather stable reaction about pH 6.3, which attended death and decomposition. In the aerated series at pH 5.0 reported in the tables the cæca were able to recover the normal reaction of pH 6.8, while in the corresponding sealed series recovery was impossible. Evidently here the first harmful attack is on respiratory activity, which is unable to eliminate carbon dioxide into acid solutions of such feeble buffer capacity without aeration. If the removal of carbon dioxide is facilitated by aeration, respiration is possible and the tissues can overcome the unfavorable conditions by neutralizing the acid.

For living starfish it was shown that the normal reaction in the cæca was about pH 6.7, for cœlomic fluid which surrounds them, pH 7.6, and for sea water pH 8.3. To change sea water from pH 8.3–6.7 would require more than a fifty times increase in carbon dioxide tension (Henderson and Cohn, 1916). On the basis of this compari-

son the starfish evidently has quite a favorable pressure gradient for carbon dioxide elimination. The optimum might then follow only in consequence of the necessity of maintaining a carbon dioxide tension necessary to overcome the resistance to elimination. But each solution was initially in carbon dioxide equilibrium with the air, and all were altered by the cæca to or well on the way toward pH 6.7. In solutions of different buffer value this required carbon dioxide tensions of quite different magnitude, so that the optimum was sought irrespective of the carbon dioxide tension it entailed. The optimum is therefore concluded to apply to processes of metabolism in general, and not to the mechanical elimination of carbon dioxide alone.

The optimum pH found is not, then, one merely favorable to respiration by the establishment of a large pressure gradient. It is an optimum, furthermore, which applies to metabolism in general. It is well known that tissues usually have optimal hydrogen ion concentrations for their most conspicuous reactions; but it did not necessarily follow that all of the metabolic processes should coincide in an optimum for the tissue as a whole, at which each individual process is most effective.

CONCLUSIONS.

The cæca maintain for themselves a hydrogen ion concentration of pH 6.7, which is different from their immediate environment, the cœlomic fluid. This in turn differs from sea water. The capacity for maintaining these differences approximately constant requires an efficient regulatory system. This does not infer the existence of a special organ or physical system in the organism, but a fact in the operation of its metabolism. Regulation of hydrogen ion concentration in living mammalian blood has proved to be an essential fact in the existence of the organism, although we cannot explain its entire operation or significance. A regulation similar in effect has been generally assumed to exist in all organisms, although it is seldom demonstrated. It is hard to conceive vital substances which could be effective and still incapable of the adjustment and preservation of their own reaction within a comparatively restricted range. It is probable that ability to regulate and maintain a constant hydrogen ion concentration agrees in general with the degree of development of the organism as a whole.

SUMMARY.

The normal reaction of the cœlomic fluid in *Patiria miniata* and *Asterias ochraceus* is pH 7.6, and of the cæca, 6.7, compared with sea water at 8.3, all without salt error correction. A medium at pH 6.7–7.0 is optimum for the cæca for ciliary survival and digestion of protein, and is maintained by carbon dioxide production. The optimum pH found for carbon dioxide production is a true one for the effect of hydrogen ion concentration on the tissue. It does not represent an elimination gradient for carbon dioxide.

Because the normal excised cæca maintain a definite hydrogen ion concentration and change their internal environment toward that as an optimum during life, there exists a regulatory process which is an important vital function.

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